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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 6 :
C12N 15/74, C07K 14/315, C12N 9/14, A23C 19/032

A1

(11) International Publication Number: **WO 95/31562**

(43) International Publication Date: 23 November 1995 (23.11.95)

(21) International Application Number: PCT/NL95/00171

(22) International Filing Date: 12 May 1995 (12.05.95)

(30) Priority Data:
94201354.1 12 May 1994 (12.05.94) EP
(34) Countries for which the regional or international application was filed: NL et al.

(71) Applicant *(for all designated States except US)*: QUEST INTERNATIONAL B.V. [NL/NL]; Huizerstraatweg 28, NL-1411 GP Naarden (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NAUTA, Arjen [NL/NL]; Helpermolenstraat 37, NL-9721 BT Groningen (NL). VENEMA, Gerard [NL/NL]; Kerklaan 26, NL-9751 NN Haren (NL). KOK, Jan [NL/NL]; Van Hamelstraat 34, NL-9714 HL Groningen (NL). LEDEBOER, Aat, M. [NL/NL]; Montignyplein 8, NL-3955 NL Rotterdam (NL).

(74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

(81) Designated States: AU, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROCESS FOR INHIBITING THE GROWTH OF A CULTURE OF LACTIC ACID BACTERIA, AND OPTIONALLY LYSING THE BACTERIAL CELLS, AND USES OF THE RESULTING LYSED CULTURE

(57) Abstract

The invention provides a process for inhibiting the growth of a culture of lactic acid bacteria, or a product containing such culture e.g. a cheese product, in which in the cells of the lactic acid bacteria a holin obtainable from bacteriophages of Gram-positive bacteria, esp. from bacteriophages of lactic acid bacteria is produced *in situ*, the gene encoding said holin being under control of a first regulatable promoter, said holin being capable of exerting a bacteriostatic effect on the cells in which it is produced by means of a system, whereby the cell membrane is perforated, while preferably the natural production of autolysin is not impaired. It is preferable that additionally a lysin obtainable from lactic acid bacteria or their bacteriophages is produced *in situ* in the cells of the lactic acid bacteria, the gene encoding said lysin being under control of a second regulatable promoter, whereby the produced lysin effects lysis of the cells of the lactic acid bacteria. The second regulatable promoter can be the same as the first regulatable promoter and the genes encoding the holin and the lysin, respectively can be placed under the same regulatable promoter in one operon. Preferably the promoters are regulatable by the food-grade ingredients or parameters. Other uses of the invention include preparing a mixture of peptides which are modified by peptidases freed after the lysis, using the lysed culture as a bactericidal agent against spoiling bacteria or pathogenic bacteria for improving the shelf life of a product containing the lysed culture.

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Process for inhibiting th growth of a culture of lactic acid bacteria, and optionally lysing th bacterial cells, and us s of th resulting lysed culture

Background of the invention and prior art

The invention relates to a process for inhibiting the growth of a culture of lactic acid bacteria, and optionally lysing the cells of said bacteria.

In this specification the following abbreviations of names of micro-organisms are used:

E. = *Escherichia*, e.g. *E. coli*,

L. = *Lactococcus*, e.g. *L. lactis*,

M. = *Micrococcus*, e.g. *M. lysodeikticus*,

S. = *Streptococcus*, e.g. *S. faecalis* and *S. pneumonia*.

In one aspect the invention relates to a process for the lysis of a culture of lactic acid bacteria, or a product containing such culture, by means of a lysin e.g. in producing a fermented food product, e.g. in cheese-making. Such a process is known from WO 90/00599 (AGRICULTURAL & FOOD RESEARCH COUNCIL (AFRC), M.J. Gasson, published 25 January 1990, ref. 1). According to that patent specification the lysin from a *Lactococcus* (preferably prolate-headed) bacteriophage was used to lyse bacterial starter cultures during cheese-making. Exemplified was the lysin of the bacteriophage ϕvML3 of *Lactococcus lactis* ML3. In particular, the lysin can be added to a cheese product or a cheese precursor mixture, e.g. after whey removal, milling and salting. However, this solution has the disadvantage that thorough mixing of the contents of the lysed cells with the cheese product is not easily obtained. Another disadvantage is that the lysin was produced by *Escherichia coli* cells, which are not food-grade. It is explicitly stated if the cell wall of the host cell is not itself degraded by the lysin then the lysin secreting transformed host may be useful in suppressing populations of bacteria which are susceptible to lysis by the lysin. Nothing is mentioned regarding addition of a transformed host cell in improving chees flavor, certainly not a transformed lactic acid bacterium.

> As an alternative it is suggested in that patent specification "to encapsulate the lysin so that the timing of its addition is not important. The encapsulating agent dissolves after the cheese-making process is complete

thus not affecting the starter bacteria before their role in acidification was complete."

This suggested alternative has the disadvantages, that (a) an encapsulating material has to be used, and (b) said material must not dissolve before the end of the cheese making process. Moreover, if the encapsulated lysin is added at the beginning of the cheese-making process, e.g. while adding the cheese starter culture to the milk, about 90% of it is removed with the whey. Thus one has to add about tenfold the required effective amount, which is economically not attractive.

In a later publication C.A. Shearman, K. Jury & M.J. Gasson (Feb. 1992, ref. 2) described an autolytic *Lactococcus lactis* expressing a cloned lactococcal bacteriophage ϕvML3 lysin gene. In particular they stated that

"(e)xpression of the cloned lysin did not impair the ability of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* strains to metabolize lactose, to clot milk and produce acid (data not shown)".

It was suggested that during the exponential phase the lysin would not, or would insufficiently be expressed. It would only be expressed in sufficient amounts to lyse an appreciable proportion of the cells during the stationary phase, which occurs at the end of the normal fermentation process. The article illustrates that maintenance of transformed lactococcal strains could be a problem. Maintenance at a temperature below 30°C slightly delayed the onset of lysis but at 30°C regrowth of lysin resistant bacteria occurred. As alternative buffering in a sucrose medium with a sucrose percentage higher than 20% was given. This does not seem to be suitable in a process of fermentation like cheese making where the fermentation step occurs at 30°C or higher and the presence of more than 20% sucrose is not acceptable.

Furthermore, at the end of that publication it was indicated that expression in the stationary phase is not completely controlled. In addition the use of osmotic buffer in a cheese maturing process is probably not very efficient timewise when taking into consideration the length of time required for a Gouda cheese immersed in a brine bath to achieve the desired degree of salt flavour the osmotic effect of salt concentration is not going to be very quick. The cheddar cheese making process would probably be more suitable as the salt addition step is more efficient, however, still requires a mixing step.

Both disclosures described the use of a lysin originating from a lactococcal bacteriophage lysin, that means an enzyme produced in nature by an undesired substance like a bacteriophage, because bacteriophage contaminations are a major problem in large scale industrial dairy fermentation processes.

In a review article R. Young (1992, ref. 4) gives a survey of the state of the art on bacteriophage lysis, both mechanism and regulation. Especially in the section "Lysis in Phage Infections of Gram-Positive Hosts" on pages 468-472 it was indicated that the DNA sequence found by Shearman c.s. (1989, ref. 5), which DNA sequence seems to be the same as that given in ref. 1, is probably not correct and that the deduced amino acid sequence might be quite different due to a mutation causing a phase shift in the reading frame. More particularly it is speculated that the DNA sequence of the lysin gene like the pneumococcal phage associated lysin genes had no signal sequence which could account for secretion across the cytoplasmic membrane, however, this was puzzling in view of the absence of a typical N-terminal signal sequence which raises the question of how the lytic enzymes escape the cytoplasm and gain access to the cell wall.

In the above mentioned review of R. Young (1992, ref. 4, especially on page 469 in the paragraph bridging both columns and pages 472-473 in the section HOLIN FAMILY) it is argued that an additional protein is required for the action of bacteriophage lysins on the cell wall of infected cells. This additional protein is required for the access of the murein hydrolase, which is the more scientific name for the bacteriophage lysin, to its murein substrate. In that review the term "holin" was used for this additional protein. It was described in that review that the holin makes perforations in the cell wall enabling the lysins to pass the membrane so that subsequently the lysins can hydrolase the murein part of the cell wall. In this specification "holin" also means a protein or peptide required for the access of a lysin to its substrate, the murein part of the cell wall.

In the Young et al review it is stated that the realignment of the Shearman sequence and assumptions of a sequencing error obscuring a start codon does present a possible basis for establishing the requirement for a holin to eff ct the release of this phage encoded murein hydrolase. Young et al. further state "If our analysis so far has taught us anything it is that any phage with a lys zyme gene should have a holin gene". This statement is however contradicted som what further on

in the same article where a lys A clone of mv1 which does not appear to possess a holin encoding sequence is illustrated as exhibiting lytic activity.

Young et al further examined the putative holin family and disclose 8 different proteins unrelated in primary sequence for which genetic or physiological evidence of holin function exists. Some postulations concerning structure and function are made, however nothing definite appears to be settled regarding this issue. They indicate that as these proteins are small, hydrophobic, without enzyme function and lethal this is an array of characteristics not likely to attract legions of biochemists. This field is thus illustrated as being quite complex with little factual knowledge and a deal of speculation.

In a publication of Ward c.s. (1993; ref. 6) it is also suggested that the sequence of Shearman *et al.* (1989; ref. 5) is probably not correct. Comparison with a very similar phage lysin gene confirmed that a frame shift in the Shearman *et al.* (ref. 5) sequence is needed for aligning the two DNA sequences. Moreover, this comparison teaches that the real phage lysin is encoded by an ORF that is probably 45 bases longer than disclosed by Shearman *et al.* (ref. 5).

C. Platteeuw and W.M. de Vos (1992, ref. 3) described the location, characterization and expression in *Escherichia coli* of lytic enzyme-encoding gene, *lytA*, of *Lactococcus lactis* bacteriophage ϕUS3. It was described that the ϕvML3 lysin, which is active on a wide range of lactococcal strains, lacked homology with known lytic enzymes. The bacteriophage ϕUS3 was identified during studying bacteriophages specific for the cheese-making strain *Lactococcus lactis* SK11 (NIZO). The results showed that the deduced amino acid sequence of LytA shares similarities with that of an autolysin of *Streptococcus pneumonia*, suggesting that the bacteriophage ϕUS3 encodes an amidase rather than a lysozyme-type muramidase. The above illustrates the difficulties facing a person skilled in the art wishing to isolate DNA-sequences from different organisms. The lack of information regarding sequences and the lack of homology between known sequences makes use of probes and primers derived from known sequences quite unlikely to lead to successful isolation of a correct DNA sequence encoding a holin from different organisms.

In EP-A2-0 510 907 (AFRC, M.J. Gasson, published 28 October 1992, ref. 7) the use of bacteriophages of food-contaminating or pathogenic bacteria or the lysins thereof to kill such bacteria was described. Examples included lysins from bacteriophages of *Listeria*

monocytogenes (phage ϕLM4) and *Clostridium tyrobutyricum* (phag ϕP). Also tests for bacterial contamination can be made specific for specific bacteria by using the appropriate bacteriophage or lysin thereof and determining whether cells are lysed thereby. That European patent thus describes the use of lysins obtained from phages of food-contaminating or even pathogenic bacteria, which is not desirable for food-grade applications. Moreover, the use of such lysins is further away from the subject of this invention, which will be discussed below as it does not lie in improving flavour of food products by autolysis of lactic acid bacteria.

In another aspect the invention relates to a process for inhibiting the growth of a culture of lactic acid bacteria without lysing the cells.

The growth of lactic acid bacteria can be inhibited in several ways.

For example, in normal fermentations with lactic acid bacteria, e.g. for the production of yoghurt, when a certain low pH is obtained the high amount of lactic acid stops further fermentation. The growth changes from the log phase to the stationary phase which in effect is some sort of inhibition of the growth.

Another possibility is that the nutrients become scarce and the so-called starvation occurs, because the necessary ingredients are no longer available for growth of the bacteria. This means that no further growth occurs.

Still another possibility is the effect of pasteurization or sterilization causing cell death.

Summary of the invention

It has now been found that holin on its own already has a bacteriostatic effect on Gramnegative bacteria like *E. coli* and Grampositive bacteria like lactic acid bacteria. Thus according to a first embodiment the invention provides a process as described in claim 1, i.e. a process for inhibiting the growth of a culture of lactic acid bacteria, which process comprises the *in situ* production in the cells of the lactic acid bacteria of a holin obtainable from bacteriophages of Gram-positive bacteria, esp. from bacteriophages of lactic acid bacteria, the gene encoding said holin being under control of a first regulatable promoter, said first regulatable promoter not normally being associat d with said holin gene, said holin being capable of exerting a

bacteriostatic effect on the cells in which it is produced by means of a system, whereby the cell membrane is perforated, while preferably the natural production of autolysin is not impaired.

According to a second embodiment the invention provides a process as described in claim 2, i.e. a process according to the first embodiment, which additionally comprises the *in situ* production in the cells of the lactic acid bacteria of a lysin obtainable from lactic acid bacteria other foodgrade grampositive microorganisms or their bacteriophages, the gene encoding said lysin being under control of a second regulatable promoter, whereby the produced lysin effects lysis of the cells of the grampositive or gramnegative bacteria, preferably the lactic acid bacteria.

Preferably the second regulatable promoter is the same as the first regulatable promoter (claim 3), and more preferably the gene encoding the holin and the gene encoding the lysin are placed under control of the same regulatable promoter in one operon (claim 4). It is advantageous for food fermentations when said first or second promoter or both can be regulated by food-grade ingredients or parameters (claim 5). The processes according to the invention can be used in the culture of lactic acid bacteria as such, but they can also be used in a product containing such culture (claim 6). A specific embodiment of this latter possibility is a process in which the lactic acid bacteria culture is used for producing a fermented food product obtainable by the fermentative action of the lactic acid bacteria and subsequently the lactic acid bacteria in the fermented food product are lysed (claim 7). A specific example of such process is one in which the fermented food product is a cheese product (claim 8). Then an additional cheese ripening step can be carried out, whereby some of the constituents after leaving the lysed cells will change the composition of the cheese product (claim 9).

A third embodiment of the invention relates to a process for combatting spoiling bacteria or pathogenic bacteria, in which a lysed culture obtained by a process according to the second embodiment of the invention is used as a bactericidal agent (claim 10). One way of use as a bactericidal agent is a process for improving the shelf life of a consumer product, in which a product obtained by a process according to either the first or the second embodiment of the invention and containing free holin or fr e lysin or both is incorporated into said consumer product in such amount that in the resulting consumer product the growth

of spoiling bacteria or pathogenic bacteria is inhibited or that their viability is strongly reduced (claim 11). Such consumer products comprise edible products, cosmetic products, and products for cleaning fabrics, hard surfaces and human skin (claim 12).

Examples of such products may be bread and bread improvers; butter, margarine and low calorie substitutes therefor; cheeses; dressings and mayonnaise-like products; meat products; food ingredients containing peptides; shampoos; creams or lotions for treatment of the human skin; soap and soap-replacement products; washing powders or liquids; and products for cleaning food production equipment and kitchen utensils.

A fourth embodiment of the invention is a process for modifying a mixture of peptides, which comprises (1) combining a culture of lactic acid bacteria with a mixture of peptides obtained by proteolysis of proteins, the cells of said culture containing both a gene encoding a holin under control of a first regulatable promoter and a gene encoding a lysin under control of a second regulatable promoter, which second and first promoter can be the same and which first and second promoter are not normally associated with the respective genes, and (2) effecting induction of the promoter or promoters for producing both the holin and the lysin in such amounts that the cells of the lactic acid bacteria are lysed and the contents of the cells containing peptidases will modify the composition of the mixture of peptides (claim 13). In order to achieve sufficient bacterium growth the host cell must not lyse too quickly, preferably lysis will occur at the end of the log phase or commencement of the stationary phase.

An alternative is a process for modifying a mixture of peptides, which comprises treating a mixture of peptides obtained by proteolysis of proteins with a lysed culture obtained by a process according to the second embodiment of the invention (claim 14).

The proteins to be proteolysed can be, for example, milk proteins or vegetable proteins, or both (claim 15).

Any of the above-mentioned processes as claimed in claims 1-15, wherein the holin is encoded by a nucleic acid sequence according to any of claims 18-20 and/or is expressed from a recombinant vector according to any of claims 21-24 and/or is expressed by a recombinant cell according to any of claims 25-27 fall within the intended scope of the invention. In addition an alternative suitable embodiment of a process according to the invention can be directed at the inducible expression of

a lysin having the amino acid sequ nce of sequence id no 7 or being a functi nal equivalent thereof.

A nucleic acid s quence encoding a holin derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium also falls within the scope of the invention. Such a nucleic acid sequence can for example encode the amino acid sequence of sequence id no 6 or a functional equivalent thereof such as the nucleic acid sequence of nucleotides 103-328 of sequence id no 5. Any nucleic acid sequence according to the invention can further be operatively linked to a first regulatable promoter, said first regulatable promoter not normally being associated with the holin encoding sequence.

Also comprised by the invention are recombinant vectors comprising any of the nucleic acid sequences in any of the claimed embodiments, said vector preferably further being foodgrade. In addition such a recombinant vector according to the invention may suitably further comprise a nucleic acid sequence encoding a lysin, both the holin and the lysin being derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium. a preferred embodiment of a recombinant vector according to the invention further comprises the natural attachment/integration system of a bacteriophage. The natural attachment/integration system of a bacteriophage can comprise the bacteriophage attachment site and an integrase gene located such that integration of the holin and optionally lysin gene will occur, said system preferably being derived from a bacteriophage that is derivable from a food grade host cell, preferably a lactic acid bacterium. A suitable recombinant vector according to the invention comprises the nucleic acid sequence encoding the holin and the nucleic acid sequence encoding the lysin operatively linked to a foodgrade inducible promoter that can be induced via a food grade mechanism. Such a promoter system can for example be a thermosensitive complex inducible promoter as is disclosed in EP94201355 and is present on plasmid pIR14. A recombinant host cell comprising a nucleic acid sequence according to any of claims 18-20 in a setting other than in its native bacteriophage and/or a recombinant vector according to any of claims 21-24 is claimed. Any of the abovementioned embodiments of recombinant host cell further comprising a nucleic acid sequence encoding a lysin, said lysin preferably being derivable from a grampositive bacterium such as a lactic

acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium, said nucleic acid sequence encoding a lysin preferably being in a setting other than in its native bacteriophage or bacterium is also suitable. Preferably a recombinant host cell according to the invention will be a food grade host cell, preferably a lactic acid bacterium. Most preferably the host cell is of the same type from which the holin and/or lysin encoding nucleic acid sequences are derived.

Brief description of the drawings

FIGURE LEGENDS belonging to the draft publication

Fig. 1. A) Schematic outline of the PCR reactions used for the amplification of *lytP*, *lytR*, and the combination of *lytP* and *lytR*. The ORF's are indicated by hatched arrows. Sequences of the amplification primers 1-4 (lyt1-lyt4) are given in Table 2 and as sequence id no 1-4 in the Sequence Listing. The scale is in kilobases (kb).

B) Schematic representation of the plasmid constructions. See for details Materials and Methods. Abbreviations: Em^R, erythromycin resistance marker; Amp^R, ampicillin resistance marker; Cm^R, chloramphenicol resistance marker; P_{spac} is a hybrid regulatory region, constructed by Yansura and Henner (29), which contains the RNA polymerase recognition sequences of an early SP01 promoter and the *lac* operator; *lacI*, *lac* repressor under the control of the *Bacillus licheniformis* penicillinase transcriptional and translational signals, indicated as P_{pen} (29); P_1 and P_2, promoters P_1 and P_2 of the bacteriophage R1-t; T, transcription terminator; ori, origin of replication; *rro*, R1-t repressor gene.

Fig. 2. Alignment of ORF 23 and the *L. lactis* subsp. *cremoris* c2 lysin (c2). Identical amino acid residues are indicated with asterisks, conserved changes by dots.

Fig. 3. Nucleotide sequence of a 1200 bp DNA fragment of R1-t carrying *lytP* and *lytR* as represented in sequence id no 5. The deduced amino acid sequences of *lytP* and *lytR* are indicated in sequence id no 6 and 7 respectively. The putative ribosomal binding sites (RBS) are underlined. Asterisks represent stop codons. The stem-loop structure downstream of *lytR* is indicated by solid arrows.

Fig. 4. Analysis of the lytic activity of the *lytR* gene product. Cell free extracts of *E. coli* cells carrying the plasmid pAG58 (lanes 1 and 2)

or pAG58R (lanes 3 and 4) were obtained two hours after the addition of IPTG. Abbreviations: ni, non-induced; i, induced. Th arrow indicates the position of a clearing zone as a result of lytic activity exhibited by the *lytR* gene product.

Fig. 5. A) Deduced amino acid sequence of the *lytP* gene product (sequence id no 6). Predicted transmembrane segments are indicated by bars, the predicted ß-turn region by t's. Charged amino acid residues are indicated + or -, depending on the sign of the charge.

B) Topological model of LytP based on the computer predictions. The membrane-spanning amino acids are indicated.

Fig. 6. A) The effect of expression of *lytP*, *lytR*, or the combination of *lytP* and *lytR* on the optical density of *E. coli* MC1000 cells. Optical density measurements of *E. coli* cells carrying either pAG58 (a), pAG58R (b), pAG58P (c) or pAG58PR (d), with (•) or without (◦) the addition of the inducer (IPTG) are indicated as a function of time. The time scale is in hours before and after the time of induction (indicated by arrow).

B) The number of colony forming units per ml of *E. coli* cells carrying either pAG58 (empty bars), pAG58R (checkered bars), pAG58P (hatched bars), or pAG58PR (filled bars) before (left diagram), and after two hours after induction (right diagram).

Fig. 7. The effect of the induced expression of *lytP*, *lytR*, or the combination of *lytP* and *lytR* on the optical density of *L. lactis* subsp. *cremoris* LL302 cells. Optical density measurements of induced *L. lactis* cells carrying either pIR12 (•), pIR1P (▵), pIR1R (▴), or pIR1PR (▫) respectively, are indicated as a function of growth. The optical density (OD) measurements of *L. lactis* carrying pIR1PR, not exposed to mitomycin C, are represented by (◦). Time scale is in hours after the time of induction with mitomycin C (1 µg/ml).

The invention is illustrated by a draft publication, which is given below.

Inducible lysis of *Lactococcus lactis* mediated by the *Lactococcus lactis* subsp. *cremoris* bacteriophage R1-t lysis functions.

SUMMARY

This work describes the involvement of two genes of the temperate *Lactococcus lactis* subsp. *cremoris* bacteriophag R1-t, *lytR* and

lytP, in the lysis of its host. The gene product of *lytR* exhibits lytic activity as it hydrolysed *Micrococcus lysodeikticus* autoclaved cell walls. The gene product f *lytP* is required in conjunction with *lytR* to obtain efficient lysis *in vivo* in *Escherichia coli* as was shown by induction studies monitoring the optical density as a measure of cell lysis: expression of *lytR* alone did not cause significant lysis of *E. coli* cells whereas simultaneous expression of *lytP* and *lytR* caused lysis of this bacterium. LytP therefore seems to have a similar function as the S protein of the *E. coli* phage lambda, *i.e.* rendering the murein substrate accessible to the lysin.

Both *lytP* and *lytR* were subcloned in an inducible expression-vector for *L. lactis*. Induction of both genes in *L. lactis* was shown to result in cell lysis as monitored by a decrease in optical density.

INTRODUCTION

Host cell lysis by temperate bacteriophages is accomplished by at least two fundamentally different mechanisms (30). The small single-stranded DNA phage ϕX174 encodes a protein which forms a channel to transport complete phage particles from the cytoplasm of the host to the environment (4, 7, 27). However, most of the known phages encode an enzyme with murein-degrading activity. These so-called lysins cause breakdown of the peptidoglycan layer which is followed by lysis of the host and the release of the phage particles.

Lysins of bacteriophages of Gram-negative bacteria so-far characterized lack a signal sequence needed for *sec*-dependent transport across the inner membrane. A second lysis function, encoded by a gene located immediately upstream of the lysin gene, is required for efficient lysis. This gene encodes a so-called holin which is believed to form holes in the cell membrane, thereby rendering the murein substrate accessible to the lysin (30).

Until recently it was believed that in Gram-positive bacteria, phage-mediated lysis was solely accomplished through the action of a phage-encoded lysin. Transit of the phage-encoded lysin across the membrane was thought to occur via the general secretory route. However, the observation that a signal sequence required for this type of transport is absent in many of the identified lysins, raised the question how the murein-degrading activity gains access to the cell wall. There is now growing support for the idea that many of these phages require a second function for lysis of their Gram-positive host. Recently it was

shown that the *Bacillus subtilis* phage φ29, gene 14, situated immediately upstream of the lysin gene, specifies a protein required for efficient release of the phag lysin to the substrat -containing environment (20). Sequence analysis of the complete *L. lactis* subsp. *cremoris* bacteriophage R1-t genome has revealed the presence of an open reading frame (ORF) similar to several lysins of other bacteriophages. In this work we show that the corresponding gene, designated *lytR*, indeed encodes a protein with cell wall degrading activity *in vitro*.

Table 1. Bacterial strains, plasmids and bacteriophage

	relevant features	reference
Bacterial strains		
L. lactis subsp. *cremoris*		
LL302	MG1363 carrying the pWV01 *repA* gene on the chromosome to ensure efficient replication	This work
E. coli		
MC1000	*ara*D139, Δ*lac*x74, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, *str*A	3
Plasmids		
pUC18	Ap^r	28
pXNB	Ap^r; pUC18 derivative, containing a 4.1-kb *Xba*I/*Nhe*I-fragment of phage R1-t	This work
pAG58	Ap^r; Cm^r	8
pAG58P	Ap^r; Cm^r; pAG58 derivative carrying *lytP*	This work
pAG58R	Ap^r; Cm^r; pAG58 derivative carrying *lytR*	This work
pAG58PR	Em^r; Cm^r; pAG58 derivative carrying *lytP* and *lytR*	This work
pUC18P	Ap^r; pUC18 derivative carrying *lytP*	This work
pUC18R	Ap^r; pUC18 derivative carrying *lytR*	This work
pUC18PR	Ap^r; pUC18 derivative carrying *lytP* and *lytR*	This work
pIR12	Em^r; carrying the regulatory region of R1-t	This work
pIR1P	Em^r; pIR12 derivative carrying *lytP*	This work
pIR1R	Em^r; pIR12 derivative carrying *lytR*	This work
pIR1PR	Em^r; pIR12 derivative carrying *lytP* and *lytR*	This work
Bacteriophage		
R1-t	type P335, small isometric lactococcal phage, isolated from *L. lactis* subsp. *cremoris* R1	9, 11

Em^r, Ap^r, and Cm^r represent resistances to erythromycin, ampicillin, and chloramphenicol, respectively.

With the us of two species-specific inducible expression systems w show that *LytR* requires an additional gene product, specified by *lytP* upstr am of *lytR*, for efficient *in vivo* lysis of *E. coli* and *L. lactis*.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, and media

The bacterial strains, phage and plasmids used in this study are listed in Table 1.

E. coli was grown in TY broth (17) or on TY broth solidified with 1.5% agar. *L. lactis* was grown in glucose M17 broth (21), or on glucose M17 agar. Erythromycin was used at 100 µg/ml and 5 µg/ml for *E. coli* and *L. lactis*, respectively. For *E. coli*, ampicillin and chloramphenicol were used at a concentration of 100 µg/ml and 5 µg/ml, respectively.

DNA techniques

Plasmid DNA was isolated essentially by the method of Birnboim and Doly (1). Restriction enzymes, Klenow enzyme, T4 DNA ligase, and T4 DNA polymerase were obtained from Boehringer GmbH (Mannheim, Germany) and used according to the instructions of the supplier. Synthetic oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.). Polymerase chain reactions were performed using Vent polymerase (New England Biolabs Inc., Beverly, MA.). Samples were heated to 94 °C for 2 min, after which target DNA was amplified in 25 subsequent cycles under the following conditions: 94 °C for 1 min; 50 °C for 2 min; 73 °C for 1 min. The primers used for amplification are listed in Table 2 and sequence id no 1-4 of the Sequence Listing. *E. coli* was used as a host for obtaining recombinant plasmids. Transformation of *E. coli* was performed by the method of Mandel and Higa (12). Plasmids were introduced in *L. lactis* subsp. *cremoris* LL302, which contains a copy of the pWV01 *repA* gene on the chromosome to ensure efficient replication, by means of electroporation (23). DNA and protein sequences were analyzed using the programs developed by Staden (19). Analysis of the LytP protein was computed with the PC/Gene program (version 6.7; IntelliGenetics, Inc., Geneva, Switzerland) using the membrane spanning domain search program SOAP, or the ß-turn search program BETATURN.

Table 2. Primers used for amplification of *lytP* and *lytR*

primer	DNA sequence (5'->3')
Lyt1	AAAACCCGGGAAGCTTGTCGACAGCAGTGATTGGTTCAACG
Lyt2	TTCTAGAAGCTTGCATGCCCCTTCTTTTTTATTATTGAC
Lyt3	AAAACCCGGGAAGCTTGTCGACGATAATACAGCAAGCCTAGTC
Lyt4	TTTTTCTAGAAGCTTGCATGCGAAGCGGGGTTAATTTATCC

*Hin*dIII (AAGCTT), *Sph*I (GCATGC), and *Sal*I (GTCGAC) restriction enzyme sites are depicted boldfaced.

IPTG and mitomycin C induction

Overnight cultures were diluted hundred-fold in fresh glucose M17 medium (*L. lactis*) or TY medium supplemented with 0.5% glucose (*E. coli*) and grown until the culture reached an OD600 of 0.3 at which point isopropyl-ß-D-thiolgalactopyranoside (IPTG) or mitomycin C (Sigma Chemical Co., St.Louis, Mo.) was added to a final concentration of 5mM or 1 µg/ml, respectively. Before the addition of IPTG, *E. coli* cells were collected by centrifugation and resuspended in an equal volume of TY medium without additional glucose.

Lytic activity assay

The lytic activity assay was performed essentially as described by Potvin *et al.* (15) with some minor adjustments as reported by Buist *et al.* (2).

Plasmid constructions

The *lytP* and *lytR* containing fragments of the R1-t genome were amplified using polymerase chain reactions (PCR's). A 4.1-kb *Xba*I/*Nhe*I fragment of the R1-t genome containing both *lytP* and *lytR* was subcloned in the unique *Xba*I site of pUC18 resulting in the plasmid pXNB. Using pXNB as a template, amplification with three different primer combinations (lyt1-lyt2, lyt3-lyt4, and lyt1-lyt4; see Fig. 1A) yielded three DNA fragments carrying ither *lytP*, *lytR*, or the combined *lytP* and *lytR*, respectively. Following digestion with *Sph*I and *Hin*dIII these three PCR products were subcloned in *Hin*dIII and *Sph*I restricted pAG58, which

resulted in plasmids pAG58P, pAG58R, and pAG58PR, carrying *lytP*, *lytR*, and the combined *lytP* and *lytR*, respectively, under the control of the IPTG inducible P_{spac} promoter (Fig. 1B). For lysis studies in *L. lactis* plasmids pAG58P, pAG58R and pAG58PR were first restricted with *Sph*I and *Sal*I. Subsequently, the DNA fragments, carrying *lytP*, *LytR* and the combination of *lytP* and *LytR*, were first subcloned in *Sph*I/*Sal*I-cut pUC18. The *Hin*dII/*Hin*dIII fragments of these three constructs, designated pUC18P, pUC18R, and pUC18PR, were cloned in the *Nru*I and *Hin*dIII sites of pIR12, resulting in the plasmids pIR1P, pIR1R, and pIR1PR, containing *lytP*, *lytR*, and both *lytP* and *lytR*, respectively, under the transcriptional control of the R1-t promoter-operator region (Fig. 4B). These plasmids were transformed to *L. lactis* subsp. *lactis* strain LL302 which contains a copy of the pWV01 *repA* gene on the chromosome to ensure efficient replication of pWV01-derived plasmids.

The construction of plasmid pIR12 was described in a co-pending application EP-94201353.3, filed on the same date entitled: Process for the lysis of a culture of lactic acid bacteria by means of a lysin, and uses of the resulting lysed culture, the specification of which is incorporated herein by reference.

RESULTS

Cloning and sequence analysis of the R1-t lysis functions.

The switch from lysogenic to lytic life cycle of the temperate *L. lactis* subsp. *cremoris* phage R1-t will ultimately result in host cell lysis caused by phage-encoded lysis function(s), followed by the release of phage particles. Inspection of the DNA sequence of R1-t revealed that ORF 23, which specifies a protein of 270 amino acids with a calculated molecular weight of 30,214 Da, shows significant similarity with the lysin genes of the *L. lactis* bacteriophages c2 (sequence id no 8) and ϕML3 (26, 18). The similarity between the deduced amino acid sequence of ORF 23 with the c2 lysin is shown in Figure 2. Moreover, ORF 23 specifies an amino acid sequence similar to the amino acid sequences of the N-terminal portions of the amidase Hbl of the *Streptococcus pneumoniae* bacteriophage HB-3 (16), and the *S. pneumoniae* LytA autolysin (5). Therefore, ORF 23, hereafter designated *lytR* (Fig. 3) (sequence id no 7), is a likely candidate for the phage-encoded lysin gene. This was not to be predicted as is apparent from the previously cited Young reference.

To test this supposition, *lytR* was cloned into the IPTG inducible expression-vector pAG58, resulting in pAG58R (Fig. 1B). Cell-

fre extracts of *E. coli* cells containing pAG58R were assayed for lytic activity on an SDS-polyacrylamide gel in which *Micrococcus lysodeikticus* autoclaved cell walls were co-polymerized. After staining of the cell wall-containing gel with methylene blue, a clearing zone is expected at positions corresponding to lytic proteins due to the breakdown of incorporated cell walls. As shown in Figure 4, in cell free extracts of pAG58-containing *E. coli* cells a clearing zone was absent at the position corresponding to a protein with the expected molecular weight of the *lytR*-encoded protein. Cell free extracts of pAG58R-containing cells, however, gave rise to a clearing zone at the expected position. A weak clearing zone was obtained with cell free extracts of uninduced cells due to limited expression of the *lytR* gene. Cell free extracts of induced pAG58R-containing cells showed an extended clearing zone, which became very large in extracts obtained two hours after induction.

According to the rules of Von Heijne (25), LytR does not seem to contain a signal sequence specific for secreted proteins using the *sec*-dependent transport system. This apparent lack of a signal sequence has also been observed in lysins of other bacteriophages of both Gram-negative and Gram-positive bacteria. For host cell lysis to occur these phages require a protein that forms holes in the cytoplasmic membrane to render the host cell peptidoglycan layer accessible to the lysin (30). ORF 22, which is situated upstream of *lytR*, specifies a protein of 75 amino acids with a predicted molecular weight of 7,688 Da (sequence id no 6). Although the predicted amino acid sequence shows no similarity with the putative hole-forming proteins of other phages, computer analysis of the protein product of ORF 22, designated hereafter as *lytP*, predicted structural similarities with these proteins. Computer analysis revealed that the protein, specified by *lytP*, has a high probability of containing a pair of transmembrane domains, separated by a sequence with a high probability of adopting a beta turn conformation (Fig. 5). In addition it contains a charged C terminus and is highly hydrophobic. Therefore, this protein might function as a pore-forming protein required for the release across the cytoplasmic membrane of the R1-t encoded LytR.

LytP* and *lytR* are required for lysis in *Escherichia coli

To determine whether the *lytP* and *lytR* gene products are involved in host cell lysis, *lytP*, *lytR*, and the combination of *lytP* and *lytR* were subcloned in the inducible expression- vector pAG58, resulting in pAG58P, pAG58R and pAG58PR, respectively (Fig. 1B). Induction studies

were performed with *E. coli* MC1000 carrying these plasmids to examine the effects of the expression of the cloned genes on the optical density of the cells (Fig. 6A). Induction of *lytR* expression did not cause any lysis of pAG58R-containing *E. coli* cells as was determined by optical density measurements. The induction of *lytP* expression, however, almost immediately halted the increase in the optical density of pAG58P-containing cells. The expression of both *lytP* and *lytR* in *E. coli* caused lysis. Lysis occurred almost immediately after the addition of IPTG to pAG58PR-containing cells, as was demonstrated by the decrease in optical density which was associated with a dramatic decrease in colony forming units (CFU's) as compared to the uninduced control (Fig. 6B). No significant difference in CFU's between cells carrying pAG58 and pAG58R was observed. However, the induction of *lytP* had a significant effect on the viability of pAG58P-containing cells. The number of CFU's dropped more than 200-fold within two hours.

Expression of *lytP* and *lytR* in *Lactococcus lactis*

In order to examine the effects of the expression of either *lytP*, *lytR* or the combined *lytP* and *lytR* on the optical density of *L. lactis* cells, plasmids pIR1P, pIR1R, and pIR1PR were constructed (Fig. 1B). Transcription of *lytP*, *lytR*, and both *lytP* and *lytR* in these plasmids is controlled by the regulatory region of phage R1-t, which incorporates the gene specifying the repressor (*rro*) of R1-t in addition to its cognate operator region (see Fig. 1B). Expression was induced by the addition of the DNA damaging substance mitomycin C. Induction studies were performed with *L. lactis* subsp. *cremoris* LL302 cells carrying the plasmids described above. Figure 7 shows that the addition of mitomycin C to *L. lactis* cells carrying pIR12 slows down the increase in optical density similar to pIR1P-containing *L. lactis* cells. The addition of mitomycin C to genetically non-modified lactococci caused lysis of a small portion of the cells (results not shown). The expression of *lytR* as well as the simultaneous expression of *lytP* and *lytR* led to a decrease in optical density, as compared to pIR12-containing cells to which mitomycin C had been added, indicating cell lysis.

DISCUSSION

We recently determined the nucleotide sequence of the temperate *L. lactis* subsp. *cremoris* bacteriophage R1-t. On the basis of the similarity of the deduced amino acid sequence with various (auto)lysins

we postulated that ORF 23, designated *lytR*, could specify the phage-encoded lysin. The *lytR* gene product would consist of 270 amino acids with an estimated molecular weight of 30,214 Da. By assaying c ll-free extracts of *E. coli* cells expressing *lytR*, it was shown that *lytR* indeed specified a protein with lytic activity.

The similarity of LytR is mainly limited to the C-terminal parts of the lysins of the lactococcal bacteriophages c2 and ϕvML3, whereas the N-terminal part of LytR is similar to the amino acid sequence of the N-terminal portion of the *S. pneumoniae* LytA autolysin. It has been proposed that LytA consists of two functional modules (16), the C-terminal domain specifying the binding site to the murein substrate and the N-terminal domain determining the specificity of the enzyme. Since LytA is an *N*-acetylmuramoyl-L-alanine amidase (6), it is tempting to speculate that LytR is also an *N*-acetylmuramoyl-L-alanine amidase.

Because of the lack of an apparent signal peptide, we hypothesized that, like many other phage-encoded lysins, LytR needs an additional factor in order to gain access to the cell wall. ORF 22, designated *lytP*, which is situated immediately upstream of *lytR*, can specify a protein of 75 amino acids with the characteristics of a so-called holin which, for other phages, was shown to render the murein substrate accessible to lysins which lack a signal peptide (30).

This hypothesis was corroborated by the observation that the expression of *lytP* is indeed needed for efficient lysis of *E. coli in vivo*. In fact, induction of *lytR* expression did not result in lysis of *E. coli*. However, *E. coli* did lyse when, in addition to *lytR*, *lytP* was also expressed. From these results it was concluded that the transit of LytR across the inner membrane is dependent on the *lytP* gene product. The induction of solely *lytP* almost immediately halted the increase in optical density and had a dramatic effect on the viability of the induced cells. This is probably caused by the spontaneous insertion of the protein into the lipid bilayer, inducing nonspecific lesions in the inner membrane and thereby dissipating the membrane potential (20). Presumably LytP forms pores in the cytoplasmic membrane, thus allowing LytR to gain access to the cell wall.

An inducible expression system for Lactococci recently developed in our laboratory , made it possible to examine the effects of expression of *lytP*, *lytR*, and th combined *lytP* and *lytR* in *L. lactis*. Expression of the combined *lytP* and *lytR* in *L. lactis* resulted in lysis of the cells. In contrast to *E. coli*, lysis was also observed when only

lytR was expressed. Lysis of cells solely expressing *lytR* is probably caused by the combined effect of mitomycin C and LytR: Since mitomycin C lyses a small proportion of the cells (results not shown), LytR is extruded in the medium, thus acting upon the cell wall from without, and masking the additional requirement for LytP to effect lysis as was the case in *E. coli*.

For bacteriophages of both Gram-negative and Gram-positive bacteria, a system based on a murein hydrolase and a second protein required for the access of the hydrolase to its murein substrate, seems to be a general phenomenon in lysis strategies. Recently it was shown that, in addition to the *B. subtilis* phage ϕ29-encoded lysin, efficient lysis of *E. coli* also required the gene 14 product. Also several lactococcal bacteriophages seem to encode an additional factor needed for host cell lysis. On the basis of structural similarity, it has been postulated that the bacteriophages c2 and ϕvML3 encode a holin (26, 30). The deduced amino acid sequence of ORF2 of the virulent bacteriophage ϕU53, isolated from *L. lactis* SK11 (14), also shares the characteristic structural traits of a holin, making it likely that it is involved in the translation of the phage-encoded lysin, LytA. This report, however, proves for the first time that a *Lactococcus*-specified holin is required for phage-induced lysis.

DRAFT PUBLICATION 2

Development of a food-grade, thermo-inducible lysis system using the regulatory region and lysis functions of the temperate *Lactococcus lactis* subsp. *cremoris* bacteriophage R1-t.

Introduction

The system is based on the food-grade removal of most of the genomic DNA of a temperate lactococcal bacteriophage in such a way that an inducible regulatory region of the temperate bacteriophage is directly placed upstream of the lysis functions encoded by the prophage. As an example of the general applicability of this strategy to any prophage with a similar genetic structure, bacteriophage R1-t was taken. To obtain the desired deletion, plasmid pBTS1 was constructed (Figure 8). In the future pBTS2 will be constructed in which *rro* is replaced by rro^{TS} and therefore can be used to make this system thermo-inducible.

Experimental procedures

Bacterial strains, phage, plasmids, and media

The bacterial strains, phage, and plasmids used in this study are listed in Table 3. *Escherichia coli* JM101 was grown in TY broth (Rottlander and Trautner, 1970) with vigorous agitation, or on TY agar, at 37 °C. When needed, ampicillin, isopropyl-ß-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl ß-galactopyranoside (X-gal) (all from Sigma Chemical Co., St. Louis, MO.) were used at concentrations of 100 µg/ml, 1mM and 0.002% (wt/vol), respectively. *L. lactis* subsp. *cremoris* was grown in M17 broth (Terzaghi and Sandine, 1975), or on M17 agar, supplemented with 0.5% glucose or lactose at 30 °C. When appropriate, erythromycin (Boehringer Mannheim, GmbH, Germany) and X-gal were used at concentrations of 5 µg/ml and 0.004% (wt/vol), respectively.

General DNA techniques and transformation

General DNA techniques were performed as described by Sambrook *et al.* (1989). Plasmid DNA was isolated by the method of Birnboim and Doly (1979) and by using QIAGEN Midi-Plasmid isolation columns (Qiagen Inc.,Chatsworth, Ca.). Restriction enzymes, alkaline phosphatase and T4 DNA ligase were obtained from Boehringer Mannheim and were used according to the instructions of the supplier. Transformation of *E. coli* was performed as described by Mandel and Higa (1970). *L. lactis* LL108 was transformed by electroporation using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.), as described by Holo and Nes (1989) with the modifications suggested by Leenhouts and Venema (1993). Electroporation of *L. lactis* R1, R131 and R1K10 was done as decribed by van der Lelie *et al.* (1988). Oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). Polymerase chain reactions (PCR's) were performed using Vent polymerase (New England Biolabs, Inc., Beverly, MA.). After heating of the samples to 94 °C for two minutes, target DNA was amplified in 30 subsequent cycles under the following conditions: 94 °C for 1 min; 50 °C for 2 min; 73 °C for 3 min. PCR fragments were purified using the QIAEX DNA Gel Extraction Kit (Qiagen Inc.).

Isolation of R1-t phage particles and DNA

An overnight culture of *L. lactis* R1 was diluted hundred-fold in 500 ml fresh lactose M17 medium and grown until the culture reached an OD600 of 0.8 at which point mitomycin C (Sigma) was added to a final concentration of 2.5 µg/ml. Incubation at 30 °C was continued in the dark until lysis occurred. Cells debris was removed by centrifugation for 10 min at 6000 rpm. Phage particles were precipitated by incubation with NaCl (0.5 M) and polyethylene glycol 6000 (10 % [wt/vol]) for three hours on ice and purified by a CsCl step gradient as described by Sambrook *et al.* (1989). The bacteriophage R1-t suspension was dialysed against several changes of 150 mM NaCl, 15 mM trisodiumcitrate. Phage DNA was obtained by extracting the suspension twice with phenol. The DNA solution was subsequently dialysed against 10 mM Tris-HCl/1 mM EDTA, pH 8,0.

Sequencing attB-sites

The attachment sites *attL* and *attR* of the bacteriophage R1-t lysogen *L. lactis* R1 were determined by means of cycle sequencing using the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit with Vent (*exo*$^-$) DNA Polymerase (Biolabs, New England). Primers attBL and attBR with flanking *Xba*I and *Pst*I sites (Table 4) were used for cloning the *attB* site of *L. lactis* MG1363. The 272-bp PCR fragment obtained with attBL and attBR was cut with *Xba*I and *Pst*I and cloned in the *Xba*I/*Pst*I sites of pUC18 and sequenced using the dideoxy-chain-termination method (Sanger *et al.*, 1977) and the T7 sequencing kit (Pharmacia AB, Uppsala, Sweden).

Phage titre determination

Supernatant taken from *L. lactis* R131 was diluted in 1 mM $MgSO_4$. The indicator strain *L. lactis* R1K10 was grown in GM17 until the OD600 was 0.7. 2 ml of culture were centrifuged and cells were resuspended in 2 ml 1 mM $MgSO_4$. An aliquot of 100 µl diluted phage-particles were added to 200 µl cells. After incubation at room temperature for 20 minutes 3 ml Top agar (0.7% GM17 agar, 0.25% glycine, 10 mM $CaCl_2$) were added, mixed, and poored on a GM17 agar-plate (1.5%) containing glycine (0.25%) and $CaCl_2$ (10 mM). The plates were incubated overnight at 30 °C and the number of plaques were determined.

Relysogenisation of L. lactis R1K10

After infection of *L. lactis* R1K10 with R1-t phage particles, turbid plaques will be picked and tested for their ability to give UV-induction of prophage. Centrifuged cells of exponentially growing cultures will be resuspended in 1 ml 1 mM $MgSO_4$ and irradiated with a Mineralight u.v. lamp (model UVG-54, 254 nm, 3.2 Jm-2s-1: Ultra-violet Products Inc.) for 10 seconds, then 1 ml 2 times GM17 + 10 mM $CaCl_2$ will be added. The culture will be incubated at 30 °C until lysis occurs.

Mitomycin C induction

Overnight cultures of *L. lactis* were diluted hundred-fold in fresh glucose M17 medium and grown until an OD600 of 0.3 at which point mitomycin C was added to a final concentration of 1 µg/ml.

Plasmid constructions

The plasmid pORIR1PR was constructed in *L. lactis* by subcloning the 2864-bp *Eco*RI/*Sph*I-fragment of pIR1PR into pORI280 restricted with *Eco*RI and *Sph*I (Figure 8). Homology analysis showed that ORF 25 of the R1-t genome shared 98% identity with the integrase-gene of bacteriophage phi LC3 (Lillehaug and Birkeland, 1993) and was therefore called *intR*. The *intR* region was amplified with flanking *Sac*I and *Xba*I sites using PCR and primers int1 and int2 (Table 4). Plasmid pUC18Int was constructed by cloning the resulting 1326-bp PCR-fragment digested with *Sac*I and *Xba*I into the *Sac*I/*Xba*I sites of pUC18. A 1047-bp *Hind*II fragment of pUC18Int, which contains the 5'-truncated *intR*, was subcloned into the alkaline phosphatase-treated *Sma*I-site of pUC18. Both the resulting plasmid pUC18Intd and pUC18Int were constructed in *E.coli* JM101 (Figure 9). The 5'-truncated *intR* was cut out of pUC18Intd with *Eco*RI and *Bam*HI and subcloned in the *Eco*RI and *Bam*HI sites of pORR1PR, resulting in pBTS1 (Figure 8). The latter construction was done in *L. lactis* LL108.

In the future *rro* will be replaced by rro^{TS} when this temperature inducible repressor is available. This will be done by replacing the 946-bp *Nco*I-*Eco*RI fragment of pBTS1 with the comparable fragment containing rro^{TS}. This will result in pBTS2. Plasmid pIR14 deposited at the Centraal Bureau voor Schimmelcultures in Baarn, The

Netherlands comprises such a temperature sensitive *rro*. A detailed description is given in European Patent Application 94201355.8.

Results

Plasmid pBTS1 was introduced in *L. lactis* LL108. As can be seen in figure 10, pBTS1 is still able to give inducible lysis after mitomycin C induction.

The initial idea was to introduce pBTS1 in *L. lactis* R1. As pBTS1 cannot replicate in *L. lactis* (it lacks the gene for the plasmid replication protein RepA) it will integrate into the chromosome of R1 under selective conditions. The integration will take place at either of three homologous regions: the *intR*-region (A), the regulatory-region (B) or the region of the lytic functions (C) (Figure 11). With appropriate primer-sets the place of integration can be distinguished (Table 5 and Figure 11). After the first recombination step in the regions A or C, a second recombination step in the region C or A, respectively, will delete the whole prophage and plasmid from the chromosome of strain R1, except for the desired functions. These two recombination steps will place the lytic functions directly under control of the regulatory region of R1-t, in a one copy situation at a well defined and stable place in the chromosome of *L. lactis*. If the integration takes place in region B (regulatory region), the second recombination step will not result in the substitution of *intR* and *rro* for the 5'-truncated *intR* and rro^{TS} (future work), respectively. The integrase deletion is needed to prevent *intR* catalysed excision.

Because of the extremely low transformation efficiency of *L. lactis* R1 (less than 1 transformant/µg pVE6007) we tried to cure the strain of its natural plasmids. We succeeded in curing two plasmids of approximately 50 kb and 2 kb, by growing *L. lactis* R1 on glucose and incubation at 37 °C. The resulting strain *L. lactis* R131 was shown by UV-induction to still contain R1-t prophage.

Sofar we have not succeeded in introducing pBTS1 into *L. lactis* R131. It appears that the prophage is induced after the electroporation. This, together with the presence of $MgCl_2$ and $CaCl_2$ in the recovery medium, makes the cells lyse (Table 6).

Therefore, we are currently trying to obtain the pBTS1 integrant by two additional strategies. Firstly, pBTS1 and pVE6007 will be introduced together in *L. lactis* R1K10. pVE6007 encodes a temperature

sensitive RepA protein enabling pBTS1 to replicate. After relysogenisation of the resulting double transformant with phage R1-t, pBTS1 will integrate into the chromosome when raising the temperature to 37 °C. The second way to obtain a 'food-grade' inducible lysis system is to introduce pBTS1 and pVE6007 together in *L. lactis* MG1363. The *attB*-region of this strain has been sequenced and appeared to have an homology of 99% with the *attB*-region of *L. lactis* R1K10 (Figure 12). *L. lactis* MG1363 will be transformed with R1-t DNA ligated at its cos-sites. After *intR*-catalysed integration of the R1-t chromosome into the attachment site of MG1363 (Figure 12), pBTS1 can integrate when raising the temperature to 37 °C.

Figure legends

Figure 8

Cloning scheme for the construction of pBTS1. *EmR*, erythromycin resistance gene; *rro*, R1-t repressor gene; *po*, promoter(p1/p2)/operator region from bacteriophage R1-t; *tec*, topological equivalent of lambda *cro*; *lytP*, R1-t holin gene; *lytR*, R1-t lysin gene; T, transcription terminator of *prtP*; ORI+, origin of replication of the lactococcal plasmid pWV01; p32, promoter sequence of ORF32 of *L. lactis*; *lacZ*, ß-galactosidase gene of *E. coli*; '*intR*, 5'-truncated R1-t integrase gene; *amp*, ampicillin resistance gene. Only relevant restriction enzyme sites are shown.

Figure 9

Cloning scheme for the construction of pUC18Intd in which the integrase gene of bacteriophage R1-t is 5'-truncated. *intR*, R1-t integrase gene; *lacZ*, ß-galactosidase gene of *E. coli*; *amp*, ampicillin resistance gene; '*intR*, 5'-truncated R1-t integrase gene. Only relevant restriction enzyme sites are shown.

Figure 10

Effect of mitomycin C on OD600 of *L. lactis* LL108 containing pORI13 (Leenhouts and Venema, 1993) (open square) and pBTS1 (filled triangle). The cultures were induced with 1 µg/ml mitomycin C at time zero (dotted line).

Figure 11

Schematic representation of the food-grade removal of most of the genomic DNA of R1-t prophage in such a way that the inducible regulatory region of the temperate bacteriophage is directly placed upstream of the lysis functions encoded by the prophage. As an example, the integration of pBTS1 in the integrase gene is depicted and the second recombination occurs in the region of the lytic functions. (A), integrase-region; (B), regulatory-region; (C), region of the lytic functions; *attR*, 'right' phage-host junction; *intR*, R1-t integrase gene; *rro*, R1-t repressor gene; *tec*, topological equivalent of lambda *cro*; PROPHAGE, genomic DNA of R1-t prophage; *lytP*, R1-t holin gene; *lytR*, R1-t lysin gene; *attL*, 'left' phage-host junction; *EmR*, erythromycin resis-

tance gene; *lacZ*, ß-galactosidase gene of *E. coli*; '*intR*, 5'-truncated R1-t integrase gene. The genes derived from the plasmid pBTS1 are shaded. The primers: attBR, R100, attBL and R40 are listed in tabl 4.

Figure 12

Comparison of the *attB* regions of *L. lactis* R1K10 and MG1363. The *attB* site is shaded. Asterisks indicate identical nucleotides.

TABLE 3. Bacterial strains, phages, and plasmid

Bacterial strain, phage or plasmid	Relevant characteristic(s)	Source or reference
Bacterial strains		
L. lactis subsp. *cremoris*		
LL108	MG1363 (plasmid-free strain) carrying the pWV01 *repA* gene, and the *Cm*^{r} gene on the chromosome (high copy)	Leenhouts, unpublished results
R1	Original R1-t lysogenic *L. lactis* subsp. *cremoris* strain	Lowrie, 1974
R131	*L. lactis* R1 cured of two natural plasmids	This study
R1K10	R1-t indicator strain	lab collection
Escherichia coli JM101	*supE thi* Δ(*lac-proAB*$^-$) [F' *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	Messing, 1979
Bacteriophage R1-t	type P335, small isometric temperate lactococcal phage, isolated from *L. lactis* subsp. *cremoris* R1	Lowrie, 1974
Plasmids		
pIR1PR	Em^r	Nauta *et al.*, 1994[a,b]
pORI280	Em^r	Leenhouts and Venema, 1993
pORIR1PR	Em^r	This study
pUC18	Ap^r	Yanisch-Perron *et al.*, 1985
pUC18Int	Ap^r	This study
pUC18Intd	Ap^r	This study
pBTS1	Em^r	This study
pVE6007	Cm^r	Maguin *et al.*, 1992
pORI13	Em^r	Leenhouts and Venema, 1993

TABLE 4. Nucleotide sequences f PCR primers

Primer	Sequence *	
int1(*SacI*)	5'-GCGCGAGCTCCCGCTCAAGTTTGACGACAAGGG-3'	(SEQ id no 9)
int2(*XbaI*)	5'-GCGCTCTAGAGGATAGATGTGCTTTAGATAATGGC-3'	(SEQ id no 10)
attBL(*XbaI*)	5'-GCGCTCTAGACAGCTATTCTATCTGTTCGTAAGGG-3'	(SEQ id no 11)
attBR(*PstI*)	5'-GCGCCTGCAGTACCTAAGCACACGAAGGCCTAGG-3'	(SEQ id no 12)
R40	5'-CAAATTGGATAGTTAAGG-3'	(SEQ id no 13)
R100	5'-CTCGTGATTACTATTGG-3'	(SEQ id no 14)

* The restriction enzyme sites are underlined.

TABLE 5. Primer-sets to check integration

Strain	Primer-sets[1, 2]	
	attBR/R100	attBL/R40
L. lactis R1	2271 bp	-
pBTS1 integrated in:		
intR-region (A)	1687 bp	-
regulatory-region (B)	2271 bp	-
lytic-region (C)	2271 bp	2249 bp
After second recomb.	1687 bp	2249 bp

[1] Primers are listed in Table 4.

[2] The sizes of the expected PCR-fragments are given.

TABLE 6. R1-t induction by electrical pulse

Electrical pulse (kV)[1]	Medium[2]	OD600 of R131[3] 1.5 hrs	4.5 hrs	21.5 hrs	PFU/ml 4.5 hrs
0	GSM17	0.323	1.097	2.248	$1.4 * 10^4$
2.5	GSM17	0.228	0.783	2.000	$4.4 * 10^5$
0	GSM17MC	0.242	0.530	1.120	$1.1 * 10^6$
2.5	GSM17MC	0.190	0.189	0.107	$1.9 * 10^7$

[1] Electroporation cuvette (2-mm electrode gap); 25 µF/200Ω

[2] Holo and Ness, 1989.

[3] 40 µl competent cells (van der Lelie et al., 1988), cells harsvested at OD600 = 0.714

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Quest International
(B) STREET: Huizerstraatweg 28
(C) CITY: Naarden
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 1411 GP
(G) TELEPHONE: 02159 - 99111
(H) TELEFAX: 02159 - 46067

(ii) TITLE OF INVENTION: Process for inhibiting the growth of a culture of lactic acid bacteria, and optionally lysing the bacterial cells, and uses of the resulting lysed culture.

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactococcus phage R1-t
(C) INDIVIDUAL ISOLATE: primer LYT1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAAACCCGGG AAGCTTGTCG ACAGCAGTGA TTGGTTCAAC G 41

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactococcus phage R1-t
(C) INDIVIDUAL ISOLATE: primer LYT2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTCTAGAAGC TTGCATGCCC CTTCTTTTTT ATTATTGAC 39

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Lactococcus phage R1-t
(C) INDIVIDUAL ISOLATE: primer LYT3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AAAACCCGGG AAGCTTGTCG ACGATAATAC AGCAAGCCTA GTC 43

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactococcus phage R1-t
(C) INDIVIDUAL ISOLATE: primer LYT4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTTTTCTAGA AGCTTGCATG CGAAGCGGGG TTAATTTATC C 41

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1200 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactococcus phage R1-t

(C) INDIVIDUAL ISOLATE: Fig.3 cds lytP and cds lytR

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 103..328

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 331..1141

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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TCTACAGGTA CATGGGAAAA TATCGGTTCA GCAGTGATTG GTTCAACGAC AATATATTAT      60

TGGAAACGAA CTGCATAAAA AATAAAAAAT AGGAGAAAGA AC ATG AAA ACA TTT        114
                                                  Met Lys Thr Phe
                                                    1

TTT AAA GAT ATG GCA GAA CGT GCC ATT AAA ACA TTT GCA CAA GCA ATG       162
Phe Lys Asp Met Ala Glu Arg Ala Ile Lys Thr Phe Ala Gln Ala Met
  5                  10                  15                  20

ATT GGC GCT TTG GGT GCT GGT GCC ACA GGC TTA ATT GGG GTT GAT TGG       210
Ile Gly Ala Leu Gly Ala Gly Ala Thr Gly Leu Ile Gly Val Asp Trp
              25                  30                  35

CTT CAA GCC TTG AGT ATC GCA GGG TTT GCA ACA GTG GTA TCA ATT CTT       258
Leu Gln Ala Leu Ser Ile Ala Gly Phe Ala Thr Val Val Ser Ile Leu
          40                  45                  50

ACT TCA TTA GCA AGT GGG ATT CCG GGC GAT AAT ACA GCA AGC CTA GTC       306
Thr Ser Leu Ala Ser Gly Ile Pro Gly Asp Asn Thr Ala Ser Leu Val
      55                  60                  65

AAT AAT AAA AAA GAA GGG GAA T AA ATG ACA ATT TAC GAC AAA ACG TTC      354
Asn Asn Lys Lys Glu Gly Glu      Met Thr Ile Tyr Asp Lys Thr Phe
  70                  75          1                   5
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CTA CTC GGC ACA GGT CAA GGT TCG TCA CAA AAG GCG AGT AAT CGA TAT 402
Leu Leu Gly Thr Gly Gln Gly Ser Ser Gln Lys Ala Ser Asn Arg Tyr
10 15 20

ATC GTG ATT CAC GAT ACC GCC AAT GAT AAT AAC CAA GGT GAT AAT AGT 450
Ile Val Ile His Asp Thr Ala Asn Asp Asn Asn Gln Gly Asp Asn Ser
25 30 35 40

GCC ACA AAT GAA GCG AGT TAT ATG CAC AAT AAC TGG CAA AAT GCC TAT 498
Ala Thr Asn Glu Ala Ser Tyr Met His Asn Asn Trp Gln Asn Ala Tyr
45 50 55

ACT CAT GCC ATT GCT GGC TGG GAT AAA GTG TAT TTG GTA GGA GAA CCT 546
Thr His Ala Ile Ala Gly Trp Asp Lys Val Tyr Leu Val Gly Glu Pro
60 65 70

GGA TAT GTT GCT TAT GGT GCA GGG AGT CCA GCT AAT GAA CGC TCA CCG 594
Gly Tyr Val Ala Tyr Gly Ala Gly Ser Pro Ala Asn Glu Arg Ser Pro
75 80 85

TTC CAA ATC GAA CTC TCT CAC TAT TCA GAC CCA GCT AAA CAA CGT TCT 642
Phe Gln Ile Glu Leu Ser His Tyr Ser Asp Pro Ala Lys Gln Arg Ser
90 95 100

TCA TAT ATC AAC TAT ATC AAT GCT GTG CGT GAA CAA GCA AAA GTA TTC 690
Ser Tyr Ile Asn Tyr Ile Asn Ala Val Arg Glu Gln Ala Lys Val Phe
105 110 115 120

GGT ATC CCT CTT ACT CTT GAT GGA GCA GGT AAT GGT ATC AAA ACT CAT 738
Gly Ile Pro Leu Thr Leu Asp Gly Ala Gly Asn Gly Ile Lys Thr His
125 130 135

AAA TGG GTT TCG GAT AAC CTT TGG GGA GAC CAT CAA GAC CCT TAC TCT 786
Lys Trp Val Ser Asp Asn Leu Trp Gly Asp His Gln Asp Pro Tyr Ser
140 145 150

TAT TTA ACA CGC ATT GGT ATT AGC AAA GAC CAA CTC GCC AAA GAC TTA 834
Tyr Leu Thr Arg Ile Gly Ile Ser Lys Asp Gln Leu Ala Lys Asp Leu
155 160 165

GCA AAC GGT ATT GGT GGG GCA TCG AAA TCT AAT CAA TCT AAT AAC GAT 882
Ala Asn Gly Ile Gly Gly Ala Ser Lys Ser Asn Gln Ser Asn Asn Asp
170 175 180

GAT TCA ACA CAC GCA ATC AAC TAC ACA CCT AAC ATG GAG GAA AAA GAA 930
Asp Ser Thr His Ala Ile Asn Tyr Thr Pro Asn Met Glu Glu Lys Glu
185 190 195 200

ATG ACT TAT CTT ATT TTT GCA AAA GAC ACT AAA CGC TGG TAC ATC ACA 978
Met Thr Tyr Leu Ile Phe Ala Lys Asp Thr Lys Arg Trp Tyr Ile Thr
205 210 215

AAC GGT ATT GAA ATC CGT TAT ATC AAA ACT GGT AGA GTT CTT GGA AAT 1026
Asn Gly Ile Glu Ile Arg Tyr Ile Lys Thr Gly Arg Val Leu Gly Asn
220 225 230

TAT CAA AAT CAA TGG TTG AAA TTC AAA CTT CCT GTG GAT ACT ATG TTC 1074
Tyr Gln Asn Gln Trp Leu Lys Phe Lys Leu Pro Val Asp Thr Met Phe
235 240 245

CAA GCA GAA GTC GAT AAA GAG TTT GGA ACT GGA GCA ACA AAT CCA AAT 1122
Gln Ala Glu Val Asp Lys Glu Phe Gly Thr Gly Ala Thr Asn Pro Asn
250 255 260

CGT GAC ATT TCA AAA GGA T AAATTAACCC CGCTTCGGCG GGTGTTTTTT 1171
Arg Asp Ile Ser Lys Gly
265 270

TAAATATAAT TTATTCAAAT AACATTTTT 1200

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Lys Thr Phe Phe Lys Asp Met Ala Glu Arg Ala Ile Lys Thr Phe
1 5 10 15

Ala Gln Ala Met Ile Gly Ala Leu Gly Ala Gly Ala Thr Gly Leu Ile
20 25 30

Gly Val Asp Trp Leu Gln Ala Leu Ser Ile Ala Gly Phe Ala Thr Val
35 40 45

Val Ser Ile Leu Thr Ser Leu Ala Ser Gly Ile Pro Gly Asp Asn Thr
50 55 60

Ala Ser Leu Val Asn Asn Lys Lys Glu Gly Glu
65 70 75

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 270 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met Thr Ile Tyr Asp Lys Thr Phe Leu Leu Gly Thr Gly Gln Gly Ser
1 5 10 15

Ser Gln Lys Ala Ser Asn Arg Tyr Ile Val Ile His Asp Thr Ala Asn
20 25 30

Asp Asn Asn Gln Gly Asp Asn Ser Ala Thr Asn Glu Ala Ser Tyr Met
35 40 45

His Asn Asn Trp Gln Asn Ala Tyr Thr His Ala Ile Ala Gly Trp Asp
50 55 60

Lys Val Tyr Leu Val Gly Glu Pro Gly Tyr Val Ala Tyr Gly Ala Gly
65 70 75 80

Ser Pro Ala Asn Glu Arg Ser Pro Phe Gln Ile Glu Leu Ser His Tyr
85 90 95

Ser Asp Pro Ala Lys Gln Arg Ser Ser Tyr Ile Asn Tyr Ile Asn Ala
100 105 110

Val Arg Glu Gln Ala Lys Val Phe Gly Ile Pro Leu Thr Leu Asp Gly
115 120 125

Ala Gly Asn Gly Ile Lys Thr His Lys Trp Val Ser Asp Asn Leu Trp
130 135 140

Gly Asp His Gln Asp Pro Tyr Ser Tyr Leu Thr Arg Ile Gly Ile Ser
145 150 155 160

Lys Asp Gln Leu Ala Lys Asp Leu Ala Asn Gly Ile Gly Gly Ala Ser
165 170 175

Lys Ser Asn Gln Ser Asn Asn Asp Asp Ser Thr His Ala Ile Asn Tyr
180 185 190

Thr Pro Asn Met Glu Glu Lys Glu Met Thr Tyr Leu Ile Phe Ala Lys
195 200 205

Asp Thr Lys Arg Trp Tyr Ile Thr Asn Gly Ile Glu Ile Arg Tyr Ile
210 215 220

Lys Thr Gly Arg Val Leu Gly Asn Tyr Gln Asn Gln Trp Leu Lys Phe
225 230 235 240

Lys Leu Pro Val Asp Thr Met Phe Gln Ala Glu Val Asp Lys Glu Phe
245 250 255

Gly Thr Gly Ala Thr Asn Pro Asn Arg Asp Ile Ser Lys Gly
260 265 270

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactococcus lactis subsp. cremoris
(C) INDIVIDUAL ISOLATE: Fig.2 c2 lysin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Leu Phe Pro Tyr Lys Lys Thr Ile Ile Ile Ile Gly Gly Gly Asn Ile
1 5 10 15

Lys Val Ser Gln Asn Gly Leu Asn Leu Ile Lys Glu Phe Glu Gly Cys
20 25 30

Arg Leu Thr Ala Tyr Lys Pro Val Pro Trp Glu Gln Met Tyr Thr Ile
35 40 45

Gly Trp Gly His Tyr Gly Val Thr Ala Gly Thr Thr Trp Thr Gln Ala
50 55 60

Gln Ala Asp Ser Gln Leu Glu Ile Asp Ile Asn Asn Lys Tyr Ala Pro
65 70 75 80

Met Val Asp Ala Tyr Val Lys Gly Lys Ala Asn Gln Asn Glu Phe Asp
85 90 95

Ala Leu Val Ser Leu Ala Tyr Asn Cys Gly Asn Val Phe Val Ala Asp
100 105 110

Gly Trp Ala Pro Phe Ser His Ala Tyr Cys Ala Ser Met Ile Pro Lys
115 120 125

Tyr Arg Asn Ala Gly Gly Gln Val Leu Gln Gly Leu Val Arg Arg Arg
130 135 140

Gln Ala Glu Leu Asn Leu Phe Asn Lys Pro Val Ser Ser Asn Ser Asn
145 150 155 160

Gln Asn Asn Gln Thr Gly Gly Met Ile Lys Met Tyr Leu Ile Ile Gly
165 170 175

Leu Asp Asn Ser Gly Lys Ala Lys His Trp Tyr Val Ser Asp Gly Val
180 185 190

Ser Val Arg His Val Arg Thr Ile Arg Met Leu Glu Asn Tyr Gln Asn
195 200 205

Lys Trp Ala Lys Leu Asn Leu Pro Val Asp Thr Met Phe Ile Ala Glu
210 215 220

Ile Glu Ala Glu Phe Gly Arg Lys Ile Asp Met Ala Ser Gly Glu Val
225 230 235 240

Lys

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C L A I M S

1. Process for inhibiting the growth of a culture of lactic acid bacteria,

which process comprises the *in situ* production in the cells of the lactic acid bacteria of a holin obtainable from bacteriophages of Gram-positive bacteria, esp. from bacteriophages of lactic acid bacteria, the gene encoding said holin being under control of a first regulatable promoter, said first regulatable promoter not normally being associated with the holin gene, said holin being capable of exerting a bacteriostatic effect on the cells in which it is produced by means of a system, whereby the cell membrane is perforated, while preferably the natural production of autolysin is not impaired.

2. Process according to claim 1, which additionally comprises the *in situ* production in the cells of the lactic acid bacteria of a lysin obtainable from grampositive bacteria, preferably lactic acid bacteria or their bacteriophages, the gene encoding said lysin being under control of a second regulatable promoter, said second regulatable promoter not normally being associated with the lysin gene whereby the produced lysin effects lysis of the cells of the lactic acid bacteria.

3. Process according to claim 2, in which the second regulatable promoter is the same as the first regulatable promoter.

4. Process according to claim 3, in which the gene encoding the holin and the gene encoding the lysin are placed under control of the same regulatable promoter in one operon.

5. Process according to claim 1 or 2, in which said first or second promoter or both are regulated by food-grade ingredients or parameters.

6. Process according to claim 1 or 2, in which the culture of lactic acid bacteria is part of a product containing such culture.

7. Process according to claim 6, in which the lactic acid bacteria culture is used for producing a fermented food product obtainable by the fermentative action of the lactic acid bacteria and subsequently the lactic acid bacteria in the fermented food product are lysed.

8. Process according to claim 7, in which the fermented food product is a cheese product.

9. Process according to claim 8, in which additi nally a cheese ripening st p is carried out, whereby some of the constituents after leaving the lysed cells will change the composition of the chees product.

10. Process for combatting spoiling bacteria or pathogenic bacteria, in which a lysed culture obtained by a proc ss as claimed in claim 2 is used as a bactericidal agent.

11. Process for improving the shelf life of a consumer product, in which a product obtained by a process as claimed in claim 1 or 2 and containing free holin or free lysin or both is incorporated into said consumer product in such amount that in the resulting consumer product the growth of spoiling bacteria or pathogenic bacteria is inhibited or that their viability is strongly reduced.

12. Process according to claim 11, in which the consumer product is selected from the group consisting of edible products, cosmetic products, and products for cleaning fabrics, hard surfaces and human skin.

13. Process for modifying a mixture of peptides, which comprises (1) combining a culture of lactic acid bacteria with a mixture of peptides obtained by proteolysis of proteins, the cells of said culture containing both a gene encoding a holin under control of a first regulatable promoter and a gene encoding a lysin under control of a second regulatable promoter, which second and first promoter can be the same, and which second and first promoters are not normally associated with the respective genes and

(2) effecting induction of the promoter or promoters for producing both the holin and the lysin in such amounts that the cells of the lactic acid bacteria are lysed and the contents of the cells containing peptidases will modify the composition of the mixture of peptides.

14. Process for modifying a mixture of peptides, which comprises treating a mixture of peptides obtained by proteolysis of proteins with a lysed culture obtained by a process according to claim 2.

15. Process according to claim 13 or 14, in which the proteins comprise milk proteins or vegetable proteins, or both.

16. Process according to any of the preceding claims, wherein the holin is encoded by a nucleic acid sequence according to any of claims 18-20 and/or is expressed from a recombinant vector according to any of claims 21-24 and/or is expressed by a recombinant cell according to any of claims 25-27.

17. Process according to any of claims 2-16, wherein the lysin has the amino acid sequence of sequence id no 7 or is a functional equivalent thereof.

18. A nucleic acid sequence encoding a holin derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium.

19. A nucleic acid sequence according to claim 18 encoding the amino acid sequence of sequence id no 6 or or a functional equivalent thereof such as the nucleic acid sequence of nucleotides 103-328 of sequence id no 5.

20. A nucleic acid sequence according to claim 18 or 19, further being operatively linked to a first regulatable promoter, said first regulatable promoter not normally being associated with the holin encoding sequence.

21 A recombinant vector comprising a nucleic acid sequence according to any of claims 18-20, said vector preferably further being foodgrade.

22. A recombinant vector according to claim 21 further comprising a nucleic acid sequence encoding a lysin, both the holin and the lysin being derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium.

23. A recombinant vector according to claim 21 or 22 further comprising a the natural attachment/integration system of a bacteriophage for example said system comprising the bacteriophage attachment site and an integrase gene located such that integration of the holin and optionally lysin gene will occur, said system preferably being derived from a bacteriophage that is derivable from a food grade host cell, preferably a lactic acid bacterium.

24. A recombinant vector according to any of the claims 21-23, wherein the nucleic acid sequence encoding the holin and the nucleic acid sequence encoding the lysin are operatively linked to a foodgrade inducible promoter that can be induced via a food grade mechanism, for example by being a thermosensitive complex inducible promoter.

25. A recombinant host cell comprising a nucleic acid sequence according to any of claims 18-20 in a setting other than in its native bacteriophage and/or a recombinant vector according to any of claims 21-24.

26. A recombinant host cell according to claim 25 further comprising a nucleic acid sequence encoding a lysin, said lysin preferably being derivable from a grampositiv bacterium such as a lactic

acid bacterium, in particular a L. lactis or a bacteriophag derivable from such a grampositive bacterium, said nucleic acid s quenc encoding a lysin preferably being in a setting other than in its native bacteriophage or bacterium.

27. A recombinant host cell according to claim 25 or 26 being a food grade host cell, preferably a lactic acid bacterium, most preferably the host cell is of the same type from which the holin and/or lysin encoding nucleic acid sequences are derived

* * * * *

Fig. 1A

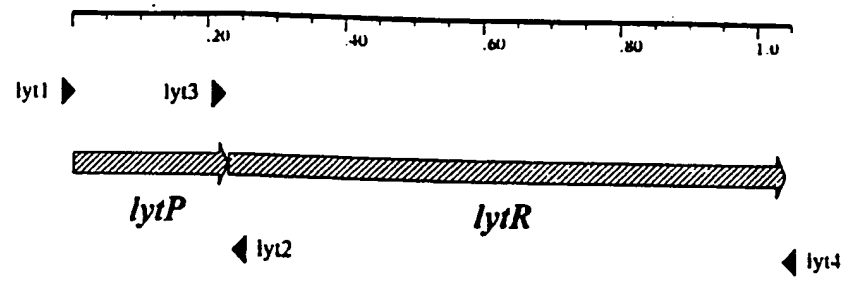

Fig. 2

```
ORF 23  MTIYDKTFLLGTGQGSSQKASNRYIVIHDTANDNNQGDNSATNEASYMHN   50
c2      LFPYKKTIII-IGGGNIKVSQNGLNLIKEFE---------GCRLTAYKPV   40
          .  * **  ..  * *    .  *   .*..              ..*

ORF 23  NWQNAYTHAIAGWDKVYLVGEPGYVAYGAGSPANERSPFQIELSHYSDPA  100
c2      PWEQMYT---IGWGHYGVTAGTTW------TQAQADSQLEIDINNKYAPM   81
          *  .  **    **  .  .  .       .*.  *   *..     *

ORF 23  KQRSSYINYINAVREQAKVFGIPLTLD-GAGNGIKTHKWVS-DNLWGDHQ  148
c2      VD-----AYVKG-KANQNEFDALVSLAYNCGNVFVADGWAPFSHAYCASM  125
                *.  .  .  *   ..*   **   .  *

ORF 23  DPYSYLTRIGISKDQLAKDLANGIGGASKSNQSNNDDSTHAINYTPNMEE  198
c2      IP-KYRNAGGQVLQGLVRRRQAELNLFNKPVSSNSNQNNQT-------GG  167
          *  *   *    *  .  .  *  **

ORF 23  KEMTYLIFAKD----TKRWYITNGIEIRYIKTGRVLGNYQNQWLKFKLPV  244
c2      MIKMYLIIGLDNSGKAKHWYVSDGVSVRHVRTIRMLENYQNKWAKLNLPV  217
           ***   *    .*.**..  *. .*  ..* *.* **** * *  ***

ORF 23  DTMFQAEVDKEFGTGATNPNRDISKG  270
c2      DTMFIAEIEAEFGRKIDMASGEVK--  241
        **** **..  ***          ..
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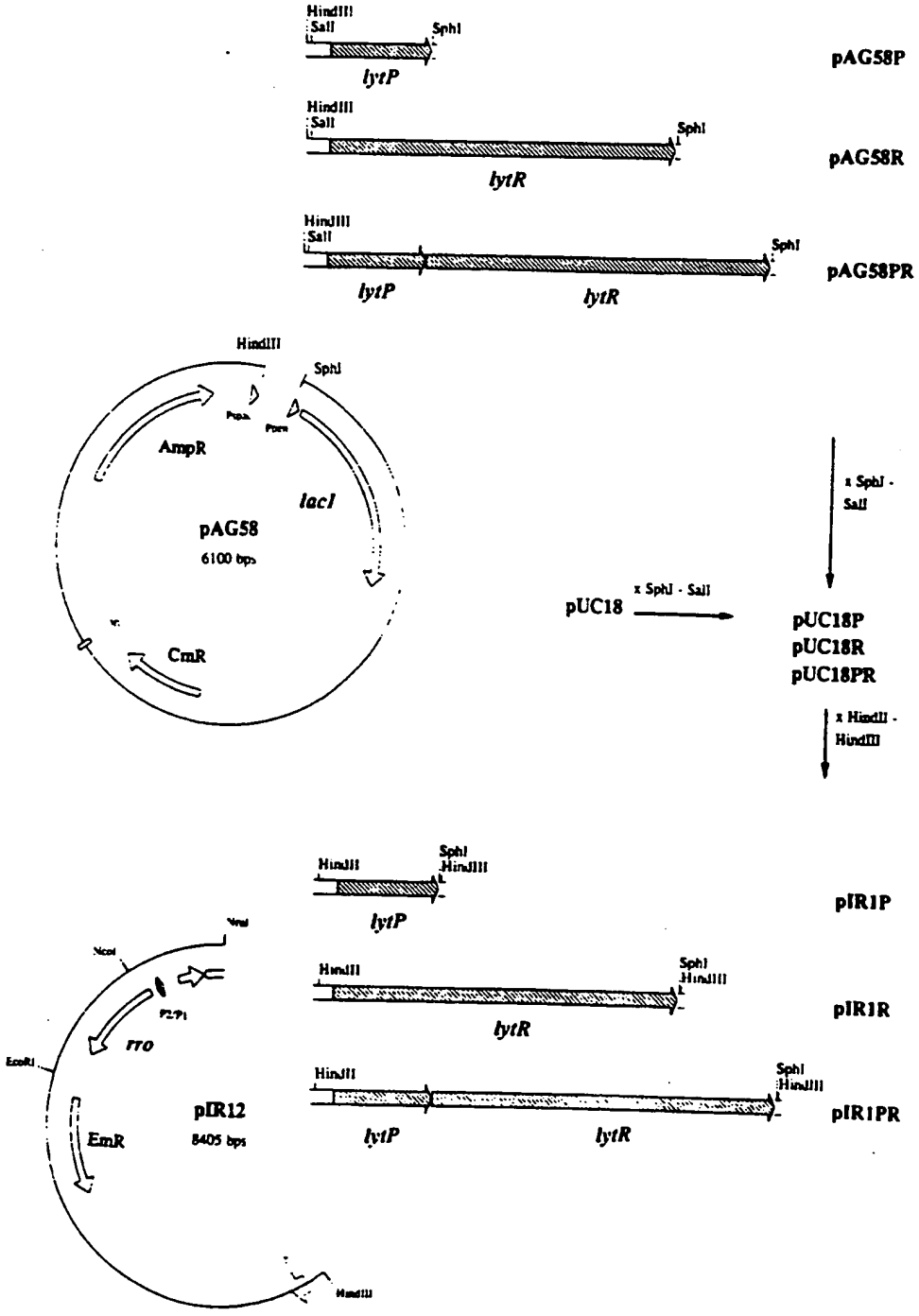
Fig. 1B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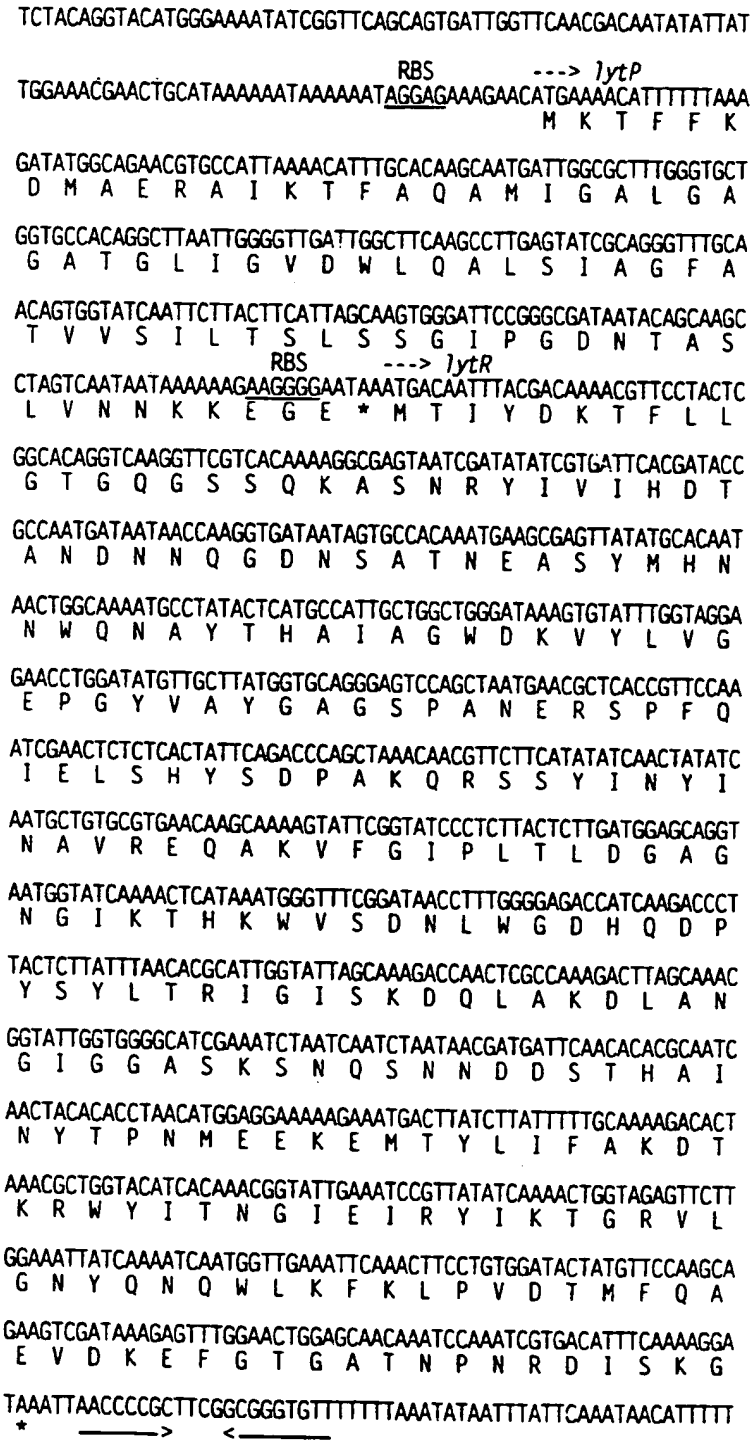
SphI
lytP
pAG58P
lytR
pAG58R
pAG58PR
AmpR
lacI
pAG58
6100 bps
CmR
x SphI - SalI
pUC18
pUC18P
pUC18R
pUC18PR
x HindII - HindIII
rro
pIR12
8405 bps
EmR
pIR1P
pIR1R
pIR1PR

Fig. 3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M K T F F K
GATATGGCAGAACGTGCCATTAAAACATTTGCACAAGCAATGATTGGCGCTTTGGGTGCT
D M A E R A I K T F A Q A M I G A L G A
GGTGCCACAGGCTTAATTGGGGTTGATTGGCTTCAAGCCTTGAGTATCGCAGGGTTTGCA
G A T G L I G V D W L Q A L S I A G F A
ACAGTGGTATCAATTCTTACTTCATTAGCAAGTGGGATTCCGGGCGATAATACAGCAAGC
T V V S I L T S L S S G I P G D N T A S
RBS ---> lytR
CTAGTCAATAATAAAAAAGAAGGGGAATAAATGACAATTTACGACAAAACGTTCCTACTC
L V N N K K E G E * M T I Y D K T F L L
GGCACAGGTCAAGGTTCGTCACAAAAGGCGAGTAATCGATATATCGTGATTCACGATACC
G T G Q G S S Q K A S N R Y I V I H D T
GCCAATGATAATAACCAAGGTGATAATAGTGCCACAAATGAAGCGAGTTATATGCACAAT
A N D N N Q G D N S A T N E A S Y M H N
AACTGGCAAAATGCCTATACTCATGCCATTGCTGGCTGGGATAAAGTGTATTTGGTAGGA
N W Q N A Y T H A I A G W D K V Y L V G
GAACCTGGATATGTTGCTTATGGTGCAGGGAGTCCAGCTAATGAACGCTCACCGTTCCAA
E P G Y V A Y G A G S P A N E R S P F Q
ATCGAACTCTCTCACTATTCAGACCCAGCTAAACAACGTTCTTCATATATCAACTATATC
I E L S H Y S D P A K Q R S S Y I N Y I
AATGCTGTGCGTGAACAAGCAAAAGTATTCGGTATCCCTCTTACTCTTGATGGAGCAGGT
N A V R E Q A K V F G I P L T L D G A G
AATGGTATCAAAACTCATAAATGGGTTTCGGATAACCTTTGGGGAGACCATCAAGACCCT
N G I K T H K W V S D N L W G D H Q D P
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Y S Y L T R I G I S K D Q L A K D L A N
GGTATTGGTGGGGCATCGAAATCTAATCAATCTAATAACGATGATTCAACACACGCAATC
G I G G A S K S N Q S N N D D S T H A I
AACTACACACCTAACATGGAGGAAAAAGAAATGACTTATCTTATTTTTGCAAAAGACACT
N Y T P N M E E K E M T Y L I F A K D T
AAACGCTGGTACATCACAAACGGTATTGAAATCCGTTATATCAAAACTGGTAGAGTTCTT
K R W Y I T N G I E I R Y I K T G R V L
GGAAATTATCAAAATCAATGGTTGAAATTCAAACTTCCTGTGGATACTATGTTCCAAGCA
G N Y Q N Q W L K F K L P V D T M F Q A
GAAGTCGATAAAGAGTTTGGAACTGGAGCAACAAATCCAAATCGTGACATTTCAAAAGGA
E V D K E F G T G A T N P N R D I S K G
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* ———> <———

Figure 4

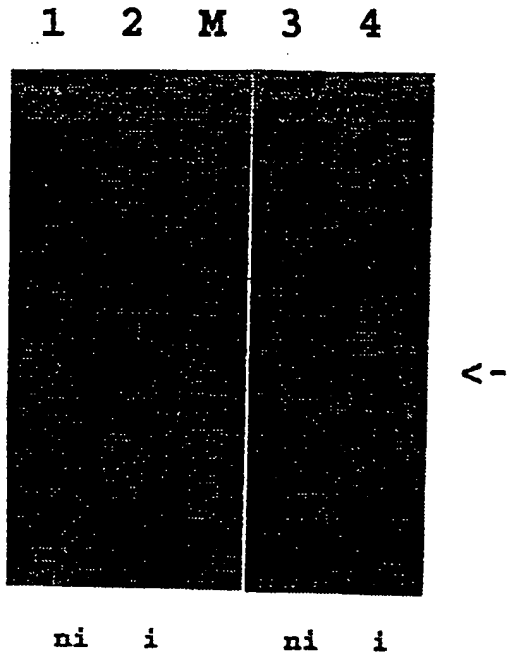
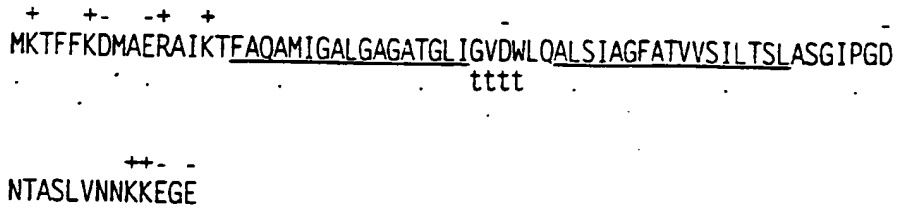


Fig.5 A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NTASLVNNKKEGE

B

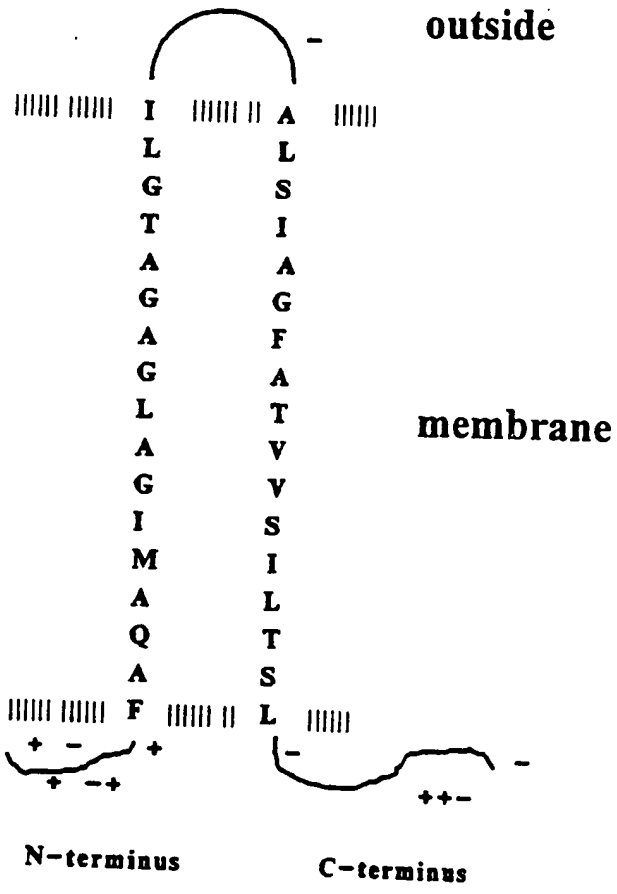
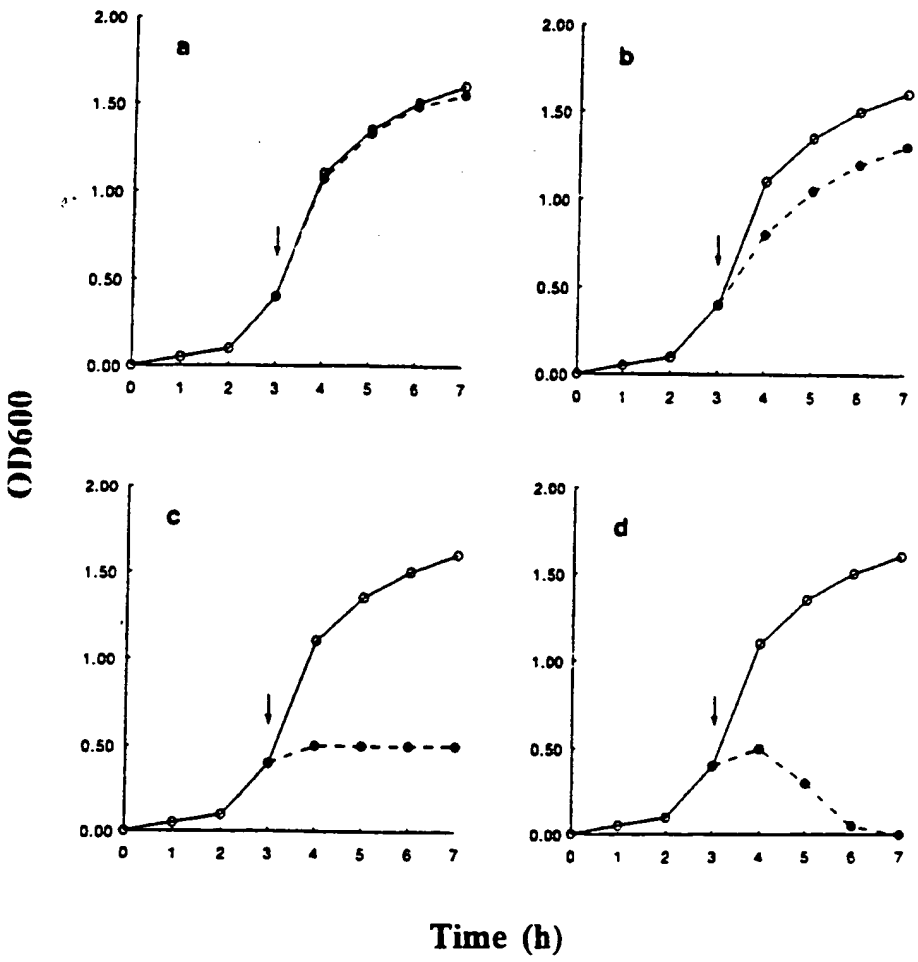

outside
membrane
inside
N-terminus
C-terminus

Fig. 6A



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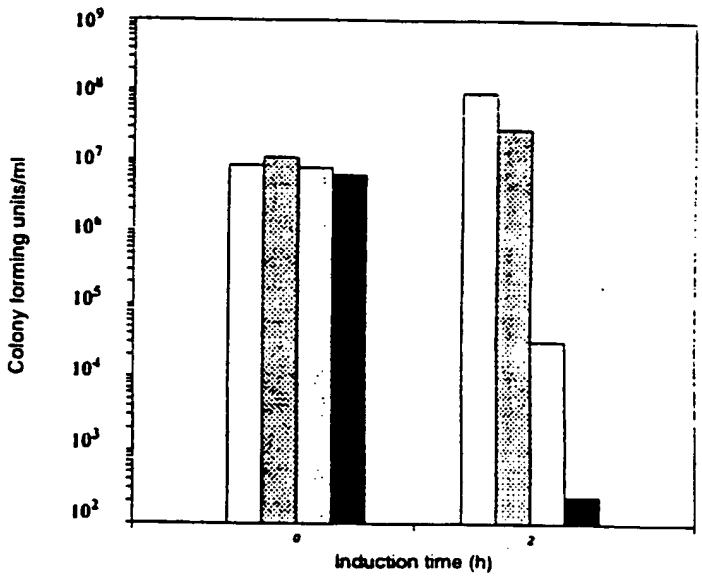
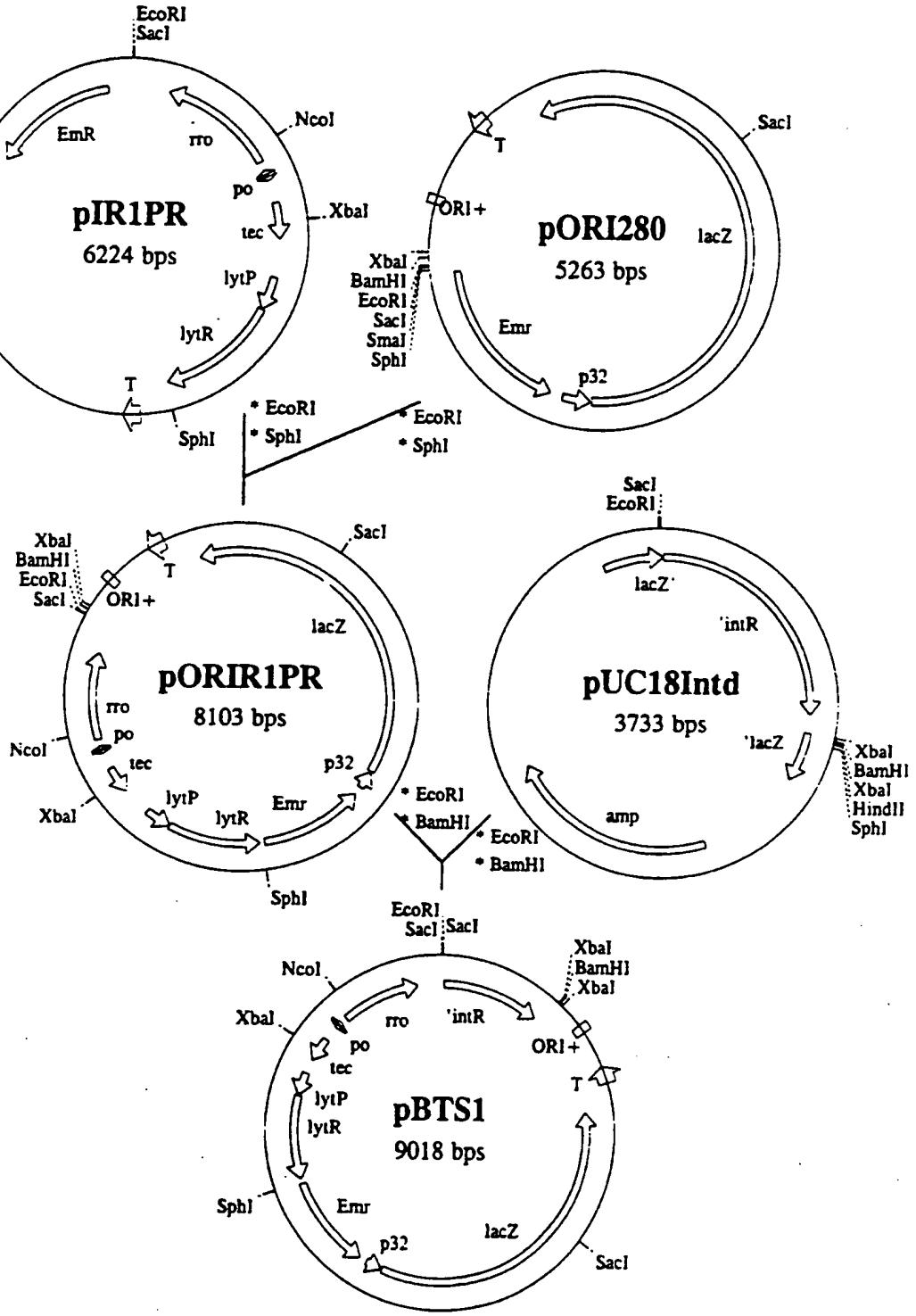


Fig. 8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SacI
EmR
rro
NcoI
po
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SphI
pORI280
5263 bps
SacI
T
ORI+
lacZ
XbaI
BamHI
EcoRI
SacI
SmaI
SphI
Emr
p32
* EcoRI
* SphI
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* SphI
pORIR1PR
8103 bps
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BamHI
EcoRI
SacI
T
SacI
ORI+
lacZ
rro
po
tec
NcoI
XbaI
lytP
lytR
Emr
p32
SphI
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3733 bps
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EcoRI
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'intR
'lacZ
amp
XbaI
BamHI
XbaI
HindII
SphI
* EcoRI
* BamHI
* EcoRI
* BamHI
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BamHI
XbaI
NcoI
XbaI
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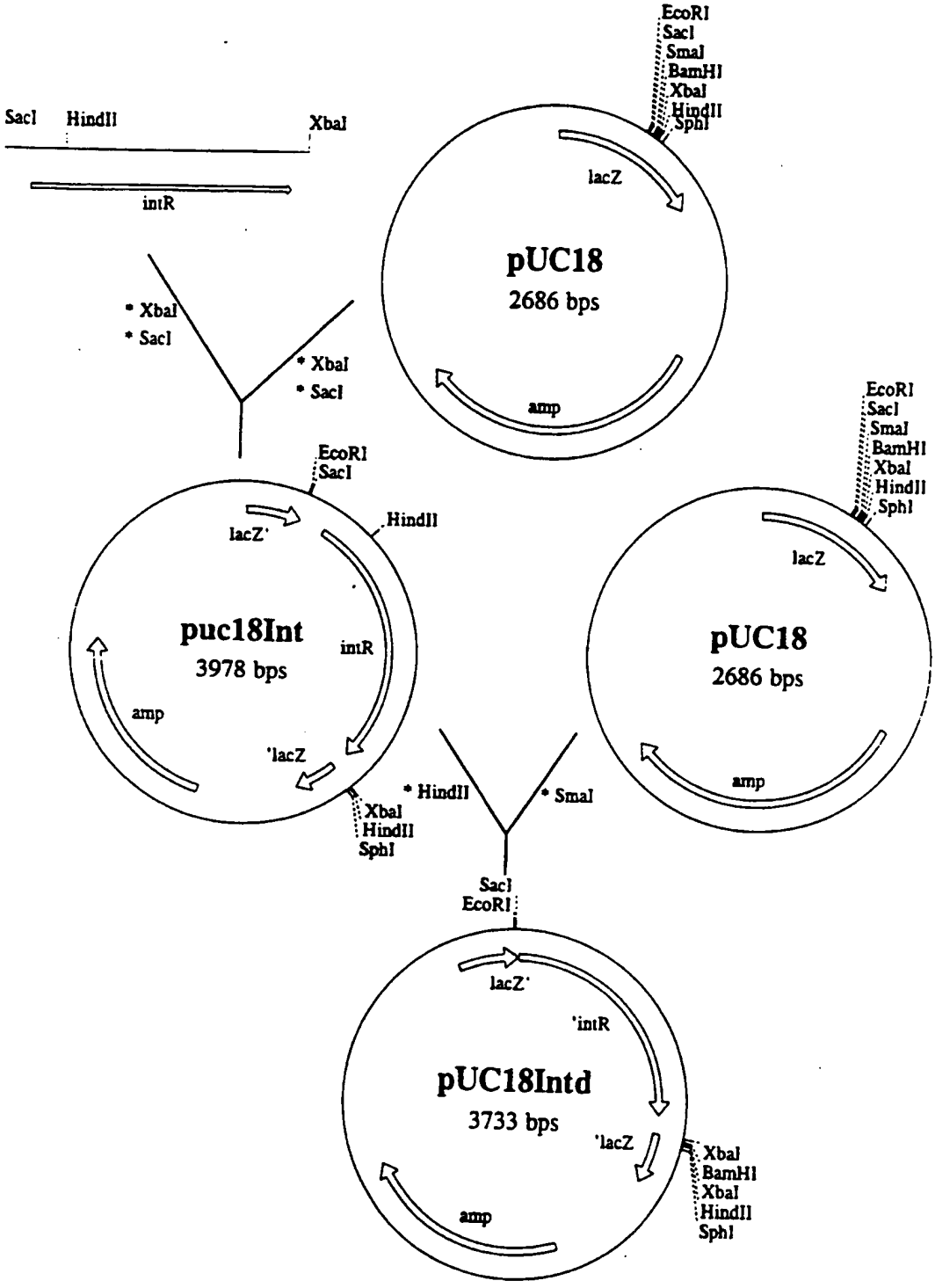
lytR
SphI
Emr
p32
lacZ
SacI

Fig. 9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* XbaI
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* XbaI
* SacI
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HindII
SphI
lacZ
pUC18
2686 bps
amp
EcoRI
SacI
lacZ'
HindII
puc18Int
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HindII
SphI
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SmaI
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* HindII
* SmaI
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Fig.10

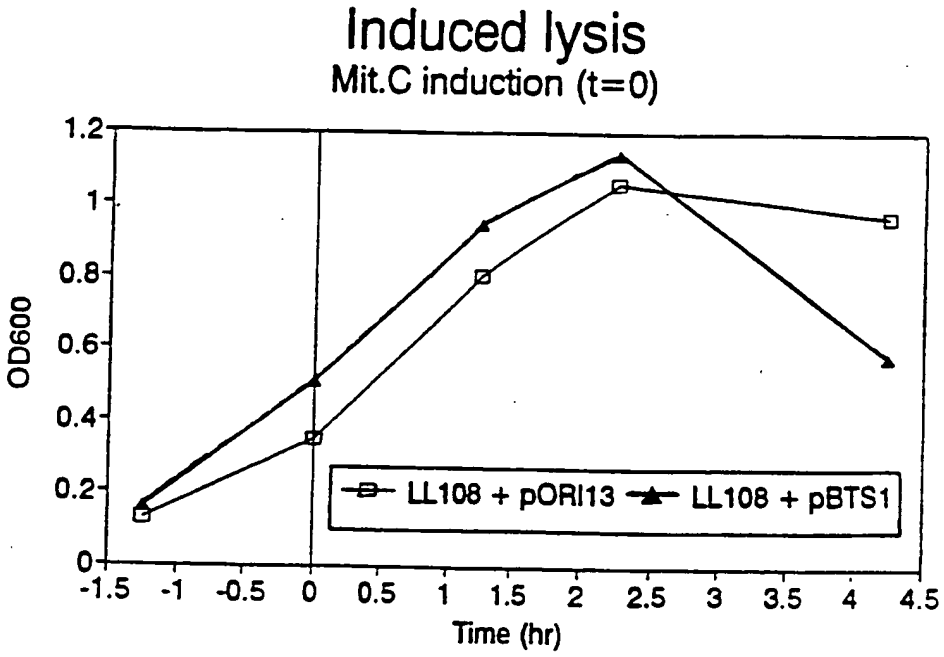


Fig.12

```
R1K10   ACAGCTATTCTATCTGTTCGTAAGGAAAACGCTCAGGAAGTGGTTGATAC 50
MG1363  ACAGCTATTCTATCTGTTCGTAAGGAAAACGCTCAGGAAGTGGTTGATAC 50
        **************************************************

RIK10   CCTTGTTAAAGCAGGAATCAAAGGCTTTCTTAACTTTGCACCTATTCGCT 100
MG1363  CCTTGTTAAAGCAGGAATCAAAGGCTTTCTTAACTTTGCACCTATTCGCT 100
        **************************************************

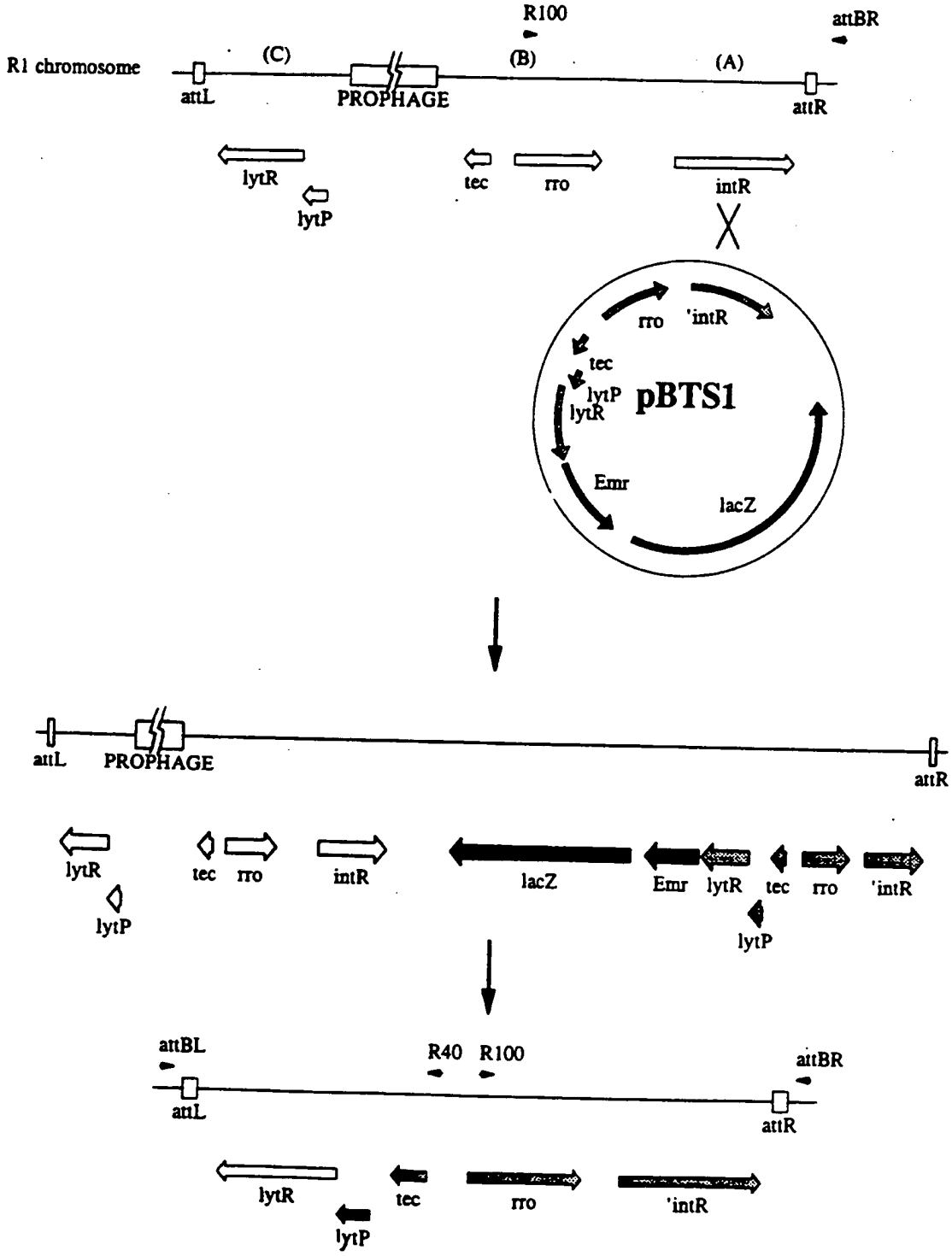
RIK10   TGAAAGTCCCTTCAGATGTTGTTGTTCAATCTATTGATTTAACTAAAGAA 150
MGI363  TGAAAGTCCCTTCAGATGTTGTTGTCCAATCTATTGATTTAACTAAAGAA 150
        ************************* *************************

R1KI0   TTGCAAACCTTGTTATTCTTCATGGGAGCTCAAGAAGAATAAAAGACAAG 200
MG1363  TTGCAAACTTTGTTATTCTTCATGGGAGCTCAAGAAGAATAAAAGACAAG 200
        ******** ***************************************

RIKI0   CAAAATTTTATCAAAACTATTAATGATACCTAGGCCTTCGTGTGCTTAGG 250
MG1363  CAAAATTTTATCAAAACTATTAATGATACCTAGGCCTTCGTGTGCTTAGG 250
        **************************************************

R1K10   TA 252
MGI363  TA 252
        **
```

Fig.11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(C)
(B)
(A)
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attR
lytR
lytP
tec
rro
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'intR
tec
lytP
lytR
pBTS1
Emr
lacZ
attL
PROPHAGE
attR
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lytP
attBL
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R100
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attR
lytR
lytP
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rro
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INTERNATIONAL SEARCH REPORT

Inter ial Application No
PCT/NL 95/00171

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/74 C07K14/315 C12N9/14 A23C19/032

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A23C C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BACTERIOLOGY, vol. 175,no. 4, 1993 pages 1038-1042, M.STEINER ET AL. 'The missing link in phage lysis of gram-positive bacteria : gene 14 of Bacillus subtilis phage sigma 29 encodes the functional homolog of lambda S protein' cited in the application	18,21
Y	see the whole document	1-17, 21-27

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 1995	17. 10. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gurdjian, D

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF GENERAL MICROBIOLOGY, vol. 138, COLCHESTER GB, page 1949 LAZAREVIC, VLADIMIR ET AL 'Sequencing and analysis of the Bacillus subtilis lytRABC divergon: a regulatory unit encompassing the structural genes of the N-acetylmuramoyl-L-alanine amidase and its modifier' see the whole document ---	1-17, 21-27
Y	BIOTECHNOLOGY, vol. 10, 1992 NEW YORK US, page 196 SHEARMAN C.A. ET AL. 'Autolytic lactococcus lactis ...' cited in the application see the whole document ---	1-17, 21-27
A	WO,A,90 00599 (AGRICULTURAL & FOOD RESEARCH COUNCIL) 25 January 1990 cited in the application see abstract; claims 1-17 ---	7-11
Y	EP,A,0 510 907 (AGRICULTURAL AND FOOD RESEARCH COUNCIL) 28 October 1992 cited in the application see claims 1-14 ---	10-12, 21-25
A	GENE., vol. 118, AMSTERDAM NL, page 115 PLATTEEUW C. ET AL. cited in the application see the whole document ---	1-27
P,X	MICROBIOLOGY, vol. 140, 1994 pages 3061-3069, C.SCHOULER ET AL. 'Sequence and organization of the Lactococcal prolate-headed bIL67 phage genome' see the whole document ---	18,21
P,X	CANADIAN J.MICROBIOL., vol. 40,no. 8, 1994 pages 658-665, N.K.BIRKELAND 'Cloning, molecular characterization, and expression of the genes encoding the lytic functions of lactococcal bacteriophage sigmaLC3 : a dual lysis system of modular design' see the whole document ---	18,21

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 60,no. 6, June 1994 pages 18785-1883, E.K.ARENDT ET AL. 'Molecular characterization of lactococcal bacteriophage Tuc 2009 and identification and analysis of genes encoding lysin, a putative holin, and two structural proteins' see the whole document	18,21

1

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Intern al Application No
PCT/NL 95/00171

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9000599	25-01-90	AU-B-	623166	07-05-92
		AU-B-	4037789	05-02-90
		EP-A-	0425572	08-05-91
		GB-A-	2243611	06-11-91
		US-A-	5360617	01-11-94
EP-A-0510907	28-10-92	GB-A-	2255561	11-11-92
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